

UCLA

UCLA Previously Published Works

Title

Decoupling the role of ROR γ t in the differentiation and effector function of TH17 cells

Permalink

<https://escholarship.org/uc/item/7vx2t7sj>

Journal

Science Advances, 8(42)

ISSN

2375-2548

Authors

Zhong, Xiancai

Wu, Hongmin

Zhang, Wencan

et al.

Publication Date

2022-10-21

DOI

10.1126/sciadv.adc9221

Copyright Information

This work is made available under the terms of a Creative Commons Attribution-NonCommercial License, available at <https://creativecommons.org/licenses/by-nc/4.0/>

Peer reviewed

GENETICS

Decoupling the role of ROR γ t in the differentiation and effector function of T_H17 cellsXiancai Zhong¹, Hongmin Wu¹, Wencan Zhang¹, Yousang Gwack², Weirong Shang³, Kyle O. Lee⁴, Noah Isakov⁵, Zhiheng He^{1*}†, Zuoming Sun^{1*}

ROR γ t is known to instruct the differentiation of T helper 17 (T_H17) cells that mediate the pathogenesis of autoimmune diseases. However, it remains unknown whether ROR γ t plays a distinct role in the differentiation and effector function of T_H17 cells. Here, we show that mutation of ROR γ t lysine-256, a ubiquitination site, to arginine (K256R) separates the ROR γ t role in these two functions. Preventing ubiquitination at K256 via arginine substitution does not affect ROR γ t-dependent thymocyte development, and T_H17 differentiation *in vitro* and *in vivo*, however, greatly impaired the pathogenesis of T_H17 cell-mediated experimental autoimmune encephalomyelitis (EAE). Mechanistically, K256R mutation impairs ROR γ t to bind to and activate *Runx1* expression critical for T_H17-mediated EAE. Thus, ROR γ t regulates the effector function of T_H17 cells in addition to T_H17 differentiation. This work informs the development of ROR γ t-based therapies that specifically target the effector function of T_H17 cells responsible for autoimmunity.

INTRODUCTION

Interleukin-17 (IL-17)-producing CD4⁺ T helper, or T helper 17, (T_H17) cells participate in immune responses against pathogens and the pathogenesis of diverse immunological diseases such as autoimmune diseases and even autism (1–6). The transcription factor retinoid-related orphan receptor γ t (ROR γ t), encoded by the gene *Rorc*, instructs the differentiation of T_H17 cells (7–9). Mutations in *Rorc* affect IL-17 production and lead to severe immune deficiency in both mouse (7) and human (10). Thus, therapies that control the pathogenic T_H17 responses are needed clinically (11, 12). Numerous pharmacological ROR γ t inhibitors have been developed for clinical application (5, 11–13). Those inhibitors are believed to prevent T_H17-dependent autoimmunity by inhibiting the generation of T_H17 cells due to the essential function of ROR γ t in T_H17 differentiation.

The strength of the T_H17 immune responses is determined by the overall number of T_H17 cells and their effector function. T_H17 cells are derived from naïve CD4⁺ T cells upon activation in the presence of an appropriate cytokine milieu including IL-6, transforming growth factor- β (TGF- β), and/or IL-23 (7, 14). Although the function of ROR γ t in T_H17 differentiation has long been demonstrated, it remains unknown whether ROR γ t plays a role in the effector function of T_H17 cells and whether ROR γ t has a distinct role in the differentiation versus effector function of T_H17 cells. In addition to regulating T_H17 cells, ROR γ t enhances thymocyte survival (15–18) and is required for lymph node development (8, 18–20). Previously, we have generated a mutation in ROR γ t that disrupts T_H17

differentiation but not thymocyte development (8), indicating that ROR γ t uses different mechanisms to regulate the function of T_H17 cells and thymocytes.

Ubiquitination is a posttranslational modification that regulates many aspects of cellular function (21). Ubiquitin is conjugated to the lysine residues of the proteins to modulate protein function by regulating protein stability and/or protein-protein interactions. Thus, cellular context-dependent ubiquitination of ROR γ t may be a mechanism to modulate the diverse ROR γ t functions. *In vitro* studies indicate the regulation of ROR γ t stability by ubiquitination (22–25). However, *in vivo* function of ROR γ t ubiquitination is difficult to prove, as it requires the generation of the mice expressing mutant ROR γ t incapable of being ubiquitinated. Previous studies used mice deficient in ubiquitin ligases or deubiquitinases to understand the role of ubiquitination in immune responses (26). Such an approach prevents the ubiquitination of all the substrates; thus, it is impossible to determine the function of a specific substrate and even less about the function of a specific ubiquitination site on the substrate in immunological function.

In this study, three *in vitro* assays were developed to dissect ROR γ t function in thymocyte development, T_H17 differentiation, and effector function in experimental autoimmune encephalomyelitis (EAE) induction. A ROR γ t mutation at a ubiquitination site, lysine (K)-256 to arginine (ROR γ t^{K256R}), was found to specifically impair the effector function of T_H17 cells in inducing EAE without interfering with ROR γ t function in T_H17 differentiation and thymocyte development. A strain of mice was established to express ROR γ t^{K256R} (ROR γ t^{K256R/K256R}), which cannot be ubiquitinated at this site. This strain of mice allows us to determine the *in vivo* function of ubiquitination of ROR γ t at a specific site. ROR γ t^{K256R/K256R} mice have normal thymocyte development, lymph node development, and T_H17 differentiation. However, ROR γ t^{K256R/K256R} mice display greatly impaired T_H17 immune responses leading to EAE. Further, ROR γ t^{K256R/K256R} T_H17 cells showed decreased ROR γ t^{K256R} binding to the promoter region of the *Runx1* gene and reduced expression of *Runx1*. Forced expression of *Runx1* in ROR γ t^{K256R/K256R} T_H17 cells restored the ability to induce EAE. Therefore, ROR γ t regulates the effector function of T_H17 cells in addition to T_H17 differentiation.

¹Department of Immunology and Theranostics, Arthur Riggs Diabetes and Metabolism Research Institute, Beckman Research Institute of the City of Hope, Duarte, CA 91010, USA. ²Department of Physiology, David Geffen School of Medicine, UCLA, Los Angeles, CA 90095, USA. ³Department of Gynecology and Obstetrics, School of Medicine, Emory University, Atlanta, GA 30322, USA. ⁴Eugene and Ruth Roberts Summer Student Academy, City of Hope, Duarte, CA 91010, USA. ⁵Department of Microbiology, Immunology, and Genetics, Ben-Gurion University of the Negev, Beer Sheva, Israel.

*Corresponding author. Email: zhe@wakehealth.edu (Z.H.); zsun@coh.org (Z.S.)

†Present address: Department of Microbiology and Immunology, Wake Forest University School of Medicine, Winston-Salem, NC 277101, USA.

RESULTS

Three assays are developed to dissect ROR γ t function

ROR γ t^{-/-} mice display defects in thymic T cell development, T_H17 differentiation, and development of T_H17-dependent EAE (7, 18). To dissect the function of ROR γ t, we developed three assays to separate these three functions. To determine the function of ROR γ t in thymocytes, we used an in vitro thymocyte differentiation system (27). CD4⁻CD8⁻ thymocytes from wild-type (WT) but not *ROR γ t*^{-/-} mice could differentiate into CD4⁺CD8⁺ and CD4⁺ cells (Fig. 1A). Consistently, *ROR γ t*^{-/-} CD4⁻CD8⁻ thymocytes transduced with a retrovirus expressing a ROR γ t (ROR γ t) but not green fluorescent protein (GFP) alone [empty virus (EV)] rescued the development of CD4⁺CD8⁺ and CD4⁺ from *ROR γ t*^{-/-} thymocyte (Fig. 1B and fig. S1A for gating strategy). We next used an in vitro assay to determine the function of ROR γ t in T_H17 differentiation. Under T_H17 polarization conditions, *ROR γ t*^{-/-} naïve CD4⁺ T cells could not differentiate into T_H17 cells unless exogenous ROR γ t was provided via retrovirus transduction (Fig. 1C and fig. S1B for gating strategy). Last, adoptive transfer EAE model enabled the testing of ROR γ t T_H17 effector function by using 2D2 T cell receptor (TCR) transgenic mice (*Tg*^{Tcr2D2}) that recognize myelin oligodendrocyte glycoprotein (MOG₃₅₋₅₅) (28, 29). *ROR γ t*^{-/-}/*Tg*^{Tcr2D2} CD4⁺ T cells were transduced with virus expressing ROR γ t, polarized under T_H17 conditions and adoptively transferred into *Rag1*^{-/-} recipients for inducing EAE (30). *ROR γ t*^{-/-}/*Tg*^{Tcr2D2} CD4⁺ T cells expressing ROR γ t induced very severe EAE (*ROR γ t*^{-/-}/*Tg*^{Tcr2D2} + ROR γ t); these mice had the highest disease score of 5 (the Institutional Animal Care and Use Committee protocol does not permit disease development beyond

this point) equivalent to *Rag1*^{-/-} recipients with WT *Tg*^{Tcr2D2} CD4⁺ T cells transduced with GFP alone (WT *Tg*^{Tcr2D2} + EV) (Fig. 1D). In contrast, *ROR γ t*^{-/-}/*Tg*^{Tcr2D2} CD4⁺ T cells expressing GFP alone (*ROR γ t*^{-/-}/*Tg*^{Tcr2D2} + EV) resulted in greatly delayed and impaired EAE with the highest disease score of 3. ROR γ t deficiency did not affect the proliferation and survival of *Tg*^{Tcr2D2} CD4⁺ T cells in vitro (fig. S1, C and D) and in vivo (fig. S1E), which is also indicated by an equivalent number CD4⁺ T cells recovered from the spleen after adoptive transfer (fig. S1F). The successful establishment of above assays allowed us to dissect three ROR γ t-regulated functions.

ROR γ t-K256, a ubiquitination site, is critical for the effector function but not the differentiation of T_H17 cells

ROR γ t has been shown to regulate T_H17 differentiation; it, however, remains unknown whether and how ROR γ t regulates the effector function of T_H17 cells. To address this question, we aimed to identify ROR γ t mutations that specifically disrupt ROR γ t function in effector T_H17 cell-mediated EAE but not in T_H17 differentiation and thymocyte development. Previously, we mutated lysine residues (K) on ROR γ t to arginine (R) to study the function of posttranslational modification of ROR γ t (8). Thus, we first compared the ability of WT and ROR γ t mutants to rescue *ROR γ t*^{-/-} thymocyte development. Only ROR γ t-K31R mutation moderately affected thymocyte development (Fig. 2A). In terms of T_H17 differentiation, K31R, K69R, and K313R impaired T_H17 differentiation (Fig. 2, B to D), consistent with our published results (8, 9, 31). Further, most ROR γ t mutations either impaired or potentiated T_H17 differentiation (Fig. 2B),

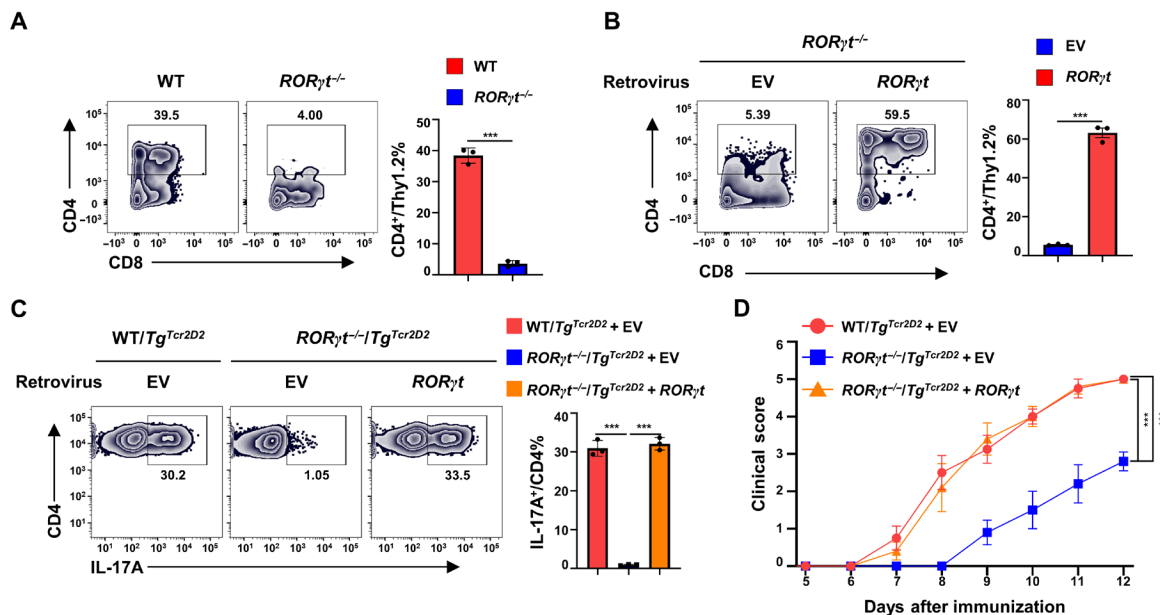


Fig. 1. Three assays are developed to dissect ROR γ t function. (A) Representative flow cytometric analysis (left) of CD4⁺ and CD8⁺ thymocytes ex vivo developed from indicated genotypes of CD4⁻CD8⁻ thymocytes placed on the OP9-DL4 stroma cells for 3 days ($n = 3$ per genotype). The number indicates the percentage of cells in the gated area throughout. Right: Percentage of CD4⁺CD8⁺ and CD4⁺ cells. (B) Representative flow cytometric analysis (left) of CD4⁺ and CD8⁺ thymocytes ex vivo developed from sorted *ROR γ t*^{-/-} CD4⁻CD8⁻ thymocytes transduced with retrovirus expressing GFP alone (EV) or with ROR γ t, and cultured on OP9-DL4 stroma cells for 3 days ($n = 3$ per genotype). Right: Percentage of CD4⁺CD8⁺ + CD4⁺ cells. (C) Representative flow cytometric analysis of IL-17A (left) and percentage of IL-17A⁺ cells (right) among indicated genotypes of CD4⁺ T cells transduced with retrovirus expressing GFP alone (EV) or with ROR γ t and polarized under T_H17 conditions for 3 days ($n = 3$ per genotype). (D) Mean clinical EAE score of *Rag1*^{-/-} mice adoptively transferred with same number of *Tg*^{Tcr2D2} or *ROR γ t*^{-/-}/*Tg*^{Tcr2D2} CD4⁺ T cells transduced with retrovirus expressing GFP alone (EV) or with ROR γ t and polarized under T_H17 conditions for 3 days. Bars are means \pm SE. *** $P < 0.001$ (two-tailed Student's t test).

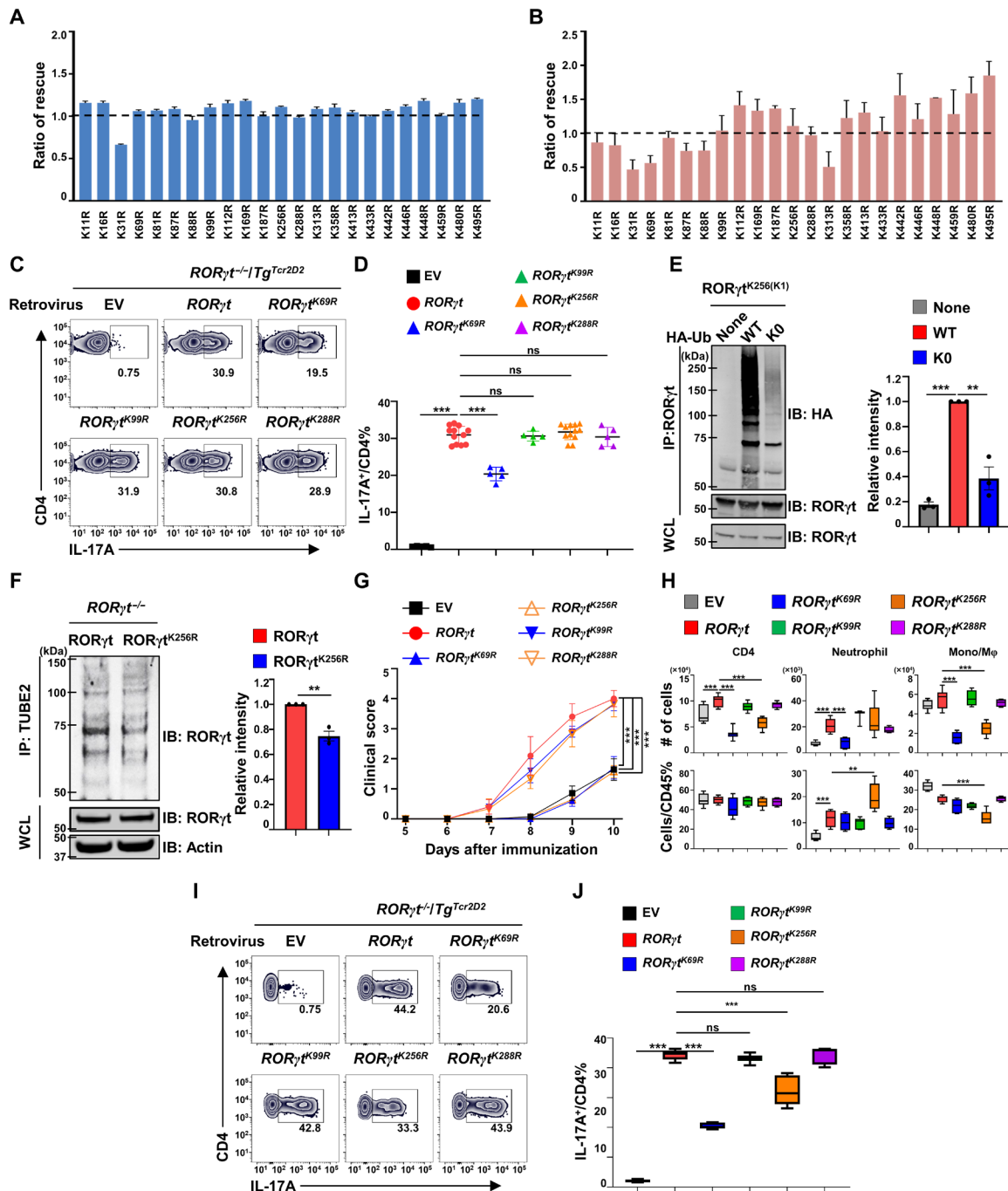


Fig. 2. RORγt-K256, a ubiquitination site, is critical for the effector function but not the differentiation of TH17 cells. (A) The ratio of rescued thymocyte development of *RORγt*^{-/-}CD4⁻CD8⁻ by indicated RORγt K-R mutants relative to that by WT RORγt (1 = 100%). (B) Ratio of rescued TH17 differentiation of *RORγt*^{-/-}CD4⁺ T cells by indicated RORγt K-R mutants relative to WT RORγt (1 = 100%). (C) Flow cytometric analysis of IL-17A⁺ cells among *RORγt*^{-/-}CD4⁺ T cells transduced with retroviruses expressing GFP alone (EV) or indicated RORγt mutants and subsequently polarized for 3 days under TH17 conditions. (D) Percentages of IL-17A⁺ cells rescued by indicated RORγt mutants shown in (C) (n = 5 to 12). (E) Immunoblot (IB) of ubiquitinated (Ub) RORγt^{K256(K1)} immunoprecipitated (IP) from human embryonic kidney (HEK) 293T cells expressing RORγt^{K256(K1)} alone (none) or together with hemagglutinin (HA)-tagged WT or mutated ubiquitin (K0, all K mutated to R). WCL, whole-cell lysates. (F) Immunoblot of ubiquitinated WT RORγt or RORγt^{K256R} (only K256 mutated to R) enriched by Tandem Ubiquitin Binding Entities 2 (TUBE2) from *RORγt*^{-/-} TH17 cells expressing indicated RORγt type. (G) Mean clinical score of *Rag1*^{-/-} mice adoptively transferred with sorted *RORγt*^{-/-}/*TgTcr2D2* TH17 cells expressing GFP alone (EV) or with indicated RORγt (n = 5 to 7 mice per group). (H) Total number and percentage of indicated immune cells recovered from the CNS of EAE-induced mice from (G). (I) Flow cytometric analysis of IL-17A⁺ cells among CD4⁺ T cells recovered from the CNS of EAE-induced mice shown in (G). (J) Box plots show median (central line), maximum, minimum (box ends), and outliers (extended lines). *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant (P > 0.05); two-tailed Student's t test.

suggesting that ROR γ t uses very different mechanisms to regulate the function of thymocytes and T_H17 cells.

To separate ROR γ t function in T_H17 differentiation versus effector T_H17 cells, we focused on mutants that did not perturb T_H17 differentiation and thymocyte development, such as K99R, K256R, and K288R (Fig. 2, A to D). K256 was identified as a prominent ubiquitination site by mass spectrometry analysis of immunoprecipitated ROR γ t (fig. S2A). To validate the K256 ubiquitination site, we generated a ROR γ t mutant with all K mutated to R except K256 (ROR γ t^{K256(K1)}) so that only K256 can be ubiquitinated. In the presence of WT ubiquitin, ROR γ t^{K256(K1)} was ubiquitinated, whereas the ubiquitination signals were absent in the presence of a mutant ubiquitin that had all K mutated to R (K0) so that it cannot be attached to the substrates (Fig. 2E). We next used a ROR γ t mutant carrying a single K256 to R mutation (ROR γ t^{K256R}) that cannot be ubiquitinated only at the K256 site. WT ROR γ t or ROR γ t^{K256R} was then expressed in ROR γ t^{-/-} T cells under T_H17-polarizing conditions. Ubiquitinated ROR γ t was readily detected in T_H17 cells (Fig. 2F). Compared to the WT ROR γ t, ROR γ t^{K256R} had obviously reduced ubiquitination signals. ROR γ t-K256 is thus ubiquitinated in T_H17 cells.

Next, we tested the effector function of T_H17 cells in the induction of EAE. As described above (Fig. 1D), in vitro-differentiated ROR γ t^{-/-}/Tg^{Tcr2D2} T_H17 cells expressing exogenous ROR γ t, ROR γ t^{K99R}, and ROR γ t^{K288R} induced severe EAE after adoptive transfer into Rag1^{-/-} recipients (Fig. 2G), suggesting that K99R and K288R do not affect the effector function of T_H17 cells in the induction of EAE, whereas ROR γ t^{K256R}-expressing ROR γ t^{-/-}/Tg^{Tcr2D2} T_H17 cells induced greatly impaired EAE. ROR γ t^{K69R} also induced impaired EAE, as we previously observed because of reduced T_H17 differentiation (8). There was no difference in the proliferation of the ROR γ t^{-/-}/Tg^{Tcr2D2} cells expressing various ROR γ t (fig. S2B). The central nervous system (CNS) immune cell infiltrate was also analyzed (Fig. 2H and fig. S2C for gating strategy). Consistent with the impaired EAE, the total number of CD4⁺ T cells and monocytes/macrophages in the CNS was significantly reduced in recipients with ROR γ t^{K256R} or ROR γ t^{K69R} mutants, indicating reduced inflammation. In addition, recipients with ROR γ t^{K256R} and ROR γ t^{K69R} cells also showed reduced infiltrate IL-17A⁺ cells in the CNS (Fig. 2I and fig. S2D for gating strategy), whereas not obvious changes in interferon- γ -positive (IFN- γ ⁺) T_H1 cells from recipients with ROR γ t^{K256R} compared to that with WT ROR γ t were observed (fig. S2, E and F). Therefore, the ROR γ t-K256 ubiquitination site, although dispensable for T_H17 differentiation, is required for the effector function of T_H17 cells in the pathogenesis of EAE.

ROR γ t-K256R mutation does not affect ROR γ t-dependent development of thymocytes and lymph nodes

To investigate the function of ROR γ t-K256 ubiquitination in vivo, we generated homozygous mice for ROR γ t^{K256R} (ROR γ t^{K256R/K256R}) under the control of endogenous ROR γ t locus (fig. S3A for genetic engineering strategy and fig. S3B for confirming the K256R mutation by sequencing). We first examined ROR γ t-dependent thymocyte development (18–20, 32). WT ROR γ t and ROR γ t^{K256R} had the same expression patterns in thymocytes (Fig. 3A); our gene-targeting strategy thus did not affect ROR γ t^{K256R} expression. Furthermore, ROR γ t^{K256R} was as stable as WT ROR γ t (fig. S3, C to D), suggesting that K256 ubiquitination site does not affect ROR γ t stability. Thymic cellularity (Fig. 3B) and distribution of different developmental stages of thymocytes, CD4⁻CD8⁻ double-negative (DN), CD4⁺CD8⁺

double-positive (DP), and CD4⁺ or CD8⁺ single-positive thymocytes (Fig. 3C) were comparable between ROR γ t^{K256R/K256R} and WT mice, which were different from ROR γ t^{-/-} mice, suggesting normal thymocyte development in ROR γ t^{K256R/K256R} mice. Furthermore, the percentage of natural killer T (NKT) and γ δ T cells in the thymus (Fig. 3, D and E) and spleens (fig. S3, E and F) was equivalent between WT and ROR γ t^{K256R/K256R} mice. The accelerated CD4⁺CD8⁺ thymocyte apoptosis (Fig. 3F and fig. S3G for analysis of apoptotic cells) accounted for the reduced percentage of CD4⁺CD8⁺ thymocytes (Fig. 3C) and decreased thymic cellularity (Fig. 3B) in ROR γ t^{-/-} mice (18), whereas the apoptosis of CD4⁺CD8⁺ thymocytes from ROR γ t^{K256R/K256R} mice was the same as that of the WT mice (Fig. 3F). In addition, thymocytes from ROR γ t^{-/-} mice had a higher percentage of cells with >2N of DNA (Fig. 3G), indicating more cells in the DNA synthesis phase of the cell cycle (18), while thymocytes from ROR γ t^{K256R/K256R} mice did not show an increased percentage of the cells in the DNA synthesis phase compared to the WT mice. Therefore, ROR γ t-K256R mutation does not affect ROR γ t function in thymocyte development. Furthermore, unlike ROR γ t^{-/-} mice that lack all the peripheral lymph nodes (18), ROR γ t^{K256R/K256R} mice developed lymph nodes including inguinal lymph nodes (Fig. 3H) and Peyer's patches (Fig. 3I), same as the WT mice. Together, prevention of ROR γ t-K256 ubiquitination does not affect the development of thymocytes and lymph nodes.

ROR γ t^{K256R/K256R} mice have normal T_H17 differentiation but develop impaired T_H17-dependent EAE

Upon maturation in the thymus, T cells migrate to the periphery to mediate immune responses. ROR γ t^{K256R/K256R} and WT mice had comparable splenocytes (fig. S4A), CD4⁺, CD8⁺ (fig. S4B), CD62L^{hi}CD44^{lo} naïve, and CD62L^{lo}CD44^{hi} memory-like T cell counts (fig. S4, C to D). We next examined T_H17 differentiation using a GFP reporter mouse line of IL-17 (Il17a^{GFP}). We confirmed that in vitro-differentiated ROR γ t^{K256R/K256R} T_H17 cells showed comparable ROR γ t (Fig. 4A) and IL-17 (Fig. 4B) expression compared to WT cells, consistent with the notion that ROR γ t-K256R mutation does affect T_H17 differentiation (Fig. 2, B and D). In addition, ROR γ t^{K256R/K256R} mice showed a normal percentage of splenic regulatory T cells (T_{regs}) (fig. S4E) and normal differentiation of T_{regs} from naïve CD4⁺ T cells (fig. S4F). To test whether ROR γ t-K256R affects the effector function of T_H17 cells responsible for the induction of EAE, we induced EAE by immunization with MOG_{35–55} peptide. Before immunization, there were almost no T_H17 cells detected in the spleens from WT and ROR γ t^{K256R/K256R} mice (Fig. 4C, top). Six days after immunization, equivalent T_H17 (fig. S4, G and H) as well as T_H1 (fig. S4, I to J) cells were induced in spleens (fig. S4, G and I) and lymph nodes (fig. S4, H and J) of WT and ROR γ t^{K256R/K256R} mice. Again, on day 12 after immunization, when EAE symptoms started to develop, the percentage of T_H17 cells still showed no obvious difference in the spleens of WT and ROR γ t^{K256R/K256R} mice (Fig. 4C, bottom), confirming that ROR γ t-K256R does not affect the generation of T_H17 cells in vivo. However, compared to WT mice, ROR γ t^{K256R/K256R} mice developed greatly impaired EAE (Fig. 4D), supporting impaired ROR γ t^{K256R/K256R} T_H17 effector function. Consistently, adoptive transfer of in vitro-differentiated ROR γ t^{K256R/K256R} T_H17 cells also induced less severe EAE in Rag1^{-/-} recipients compared to that induced by WT T_H17 cells (fig. S4K). In addition, impaired EAE induction was associated with reduced immune cell infiltrate including CD4⁺, CD8⁺, B cells, and monocytes in the CNS of ROR γ t^{K256R/K256R} mice, although no

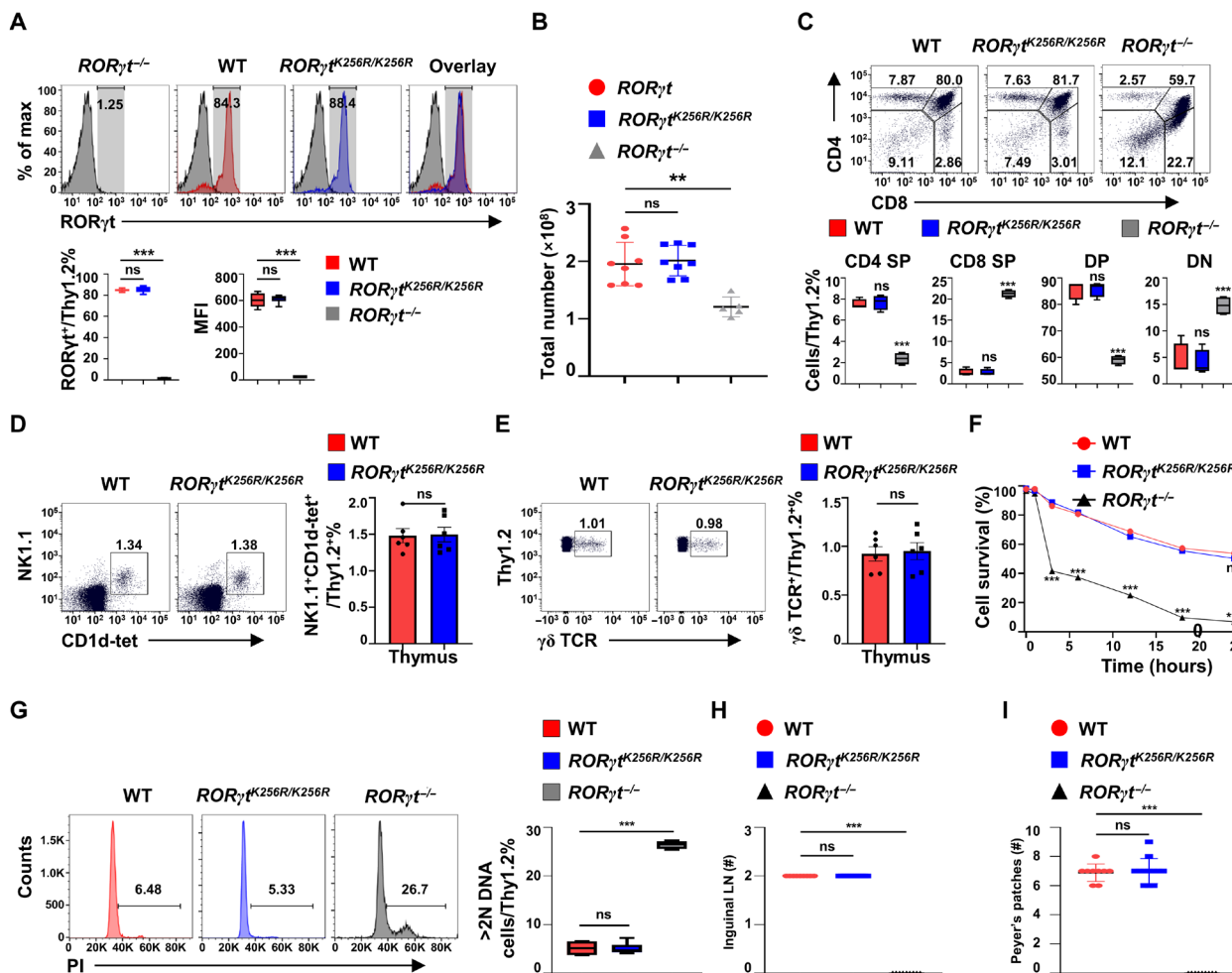


Fig. 3. ROR γ t-K256R mutation does not affect ROR γ t-dependent development of thymocytes and lymph nodes. (A) Flow cytometric analysis of ROR γ t in thymocytes obtained from indicated mice ($n = 5$ to 8). MFI, mean fluorescence intensity. (B) Total thymocyte numbers of the indicated mice ($n = 5$ to 8). The graph shows mean \pm SD. (C) Flow cytometric analysis of CD4 and CD8 on thymocytes from indicated mice ($n = 4$ per genotype). (D) Representative flow cytometric analysis of NKT cells ($n = 4$) from the thymus of the indicated mice. (E) Representative flow cytometric analysis of $\gamma\delta$ T cells ($n = 4$) from the thymus of the indicated mice. (F) Flow cytometric analysis of the survival of DP thymocytes cultured in vitro for the indicated time ($n = 4$ per genotype). (G) Representative flow cytometric analysis of DNA content of indicated thymocytes stained by propidium iodide (PI) ($n = 4$ to 6 per genotype). (H and I) Number of inguinal lymph nodes (LN) (H) and Peyer's patches (I) in the indicated mice ($n = 9$ to 12 per genotype). $**P < 0.01$; $***P < 0.001$ (two-tailed t test). (A, C, E, F, and G) Box plots or scatter plots show median (central line), maximum, minimum (box ends), and outliers (extended lines).

obvious changes were observed in neutrophil numbers (Fig. 4E and fig. S4L for gating strategy). Further analysis of CNS lymphocyte infiltrate in the $ROR\gamma t^{K256R/K256R}$ mice showed a decreased percentage of IL-17A⁺CD4⁺ cells (Fig. 4F), particularly pathogenic IL-17A⁺ granulocyte-macrophage colony-stimulating factor-positive (GM-CSF⁺) cells that play an important role in EAE development (Fig. 4, G and H, and fig. S4M for gating strategy) (2, 3). These results suggest that ROR γ t-K256R mutation, which prevents the ubiquitination, impairs the effector function but not the differentiation of T_H17 cells responsible for the pathogenesis of EAE.

ROR γ t-K256R impairs the pathways critical for the pathogenesis of EAE but not for the T_H17 differentiation

To understand the mechanisms responsible for the ROR γ t-K256R mutation-disrupted effector function of T_H17 cells responsible for

EAE, GFP⁺ T_H17 cells derived from WT and $ROR\gamma t^{K256R/K256R}/IL-17^{GFP}$ CD4⁺ T cells were sorted to high purity (>98%) (fig. S5A for gating strategy) and subjected to RNA sequencing (RNA-seq) analysis. On the basis of the computational principal components analysis, the six RNA-seq samples were divided into two groups: WT and $ROR\gamma t^{K256R/K256R}$ T_H17 cells (fig. S5B), indicating reproducible gene expression patterns within each group and thus the high quality of RNA-seq results. We identified 1375 differentially expressed genes (DEGs) [$P < 0.05$ and fold change (FC) > 1.5], with 943 up-regulated and 432 down-regulated genes, between WT and $ROR\gamma t^{K256R/K256R}$ T_H17 cells (Fig. 5A, fig. S5C, and tables S2 and S3). Subjection of DEGs to pathway analysis did not find significant changes in the T_H17 differentiation pathway (Fig. 5, B and C) between WT and $ROR\gamma t^{K256R/K256R}$ cells, confirming that ROR γ t-K256R mutation does not affect T_H17 differentiation. Down-regulated pathways in $ROR\gamma t^{K256R/K256R}$ cells include IL-23 signaling (Fig. 5, B and D) and glycolysis pathways

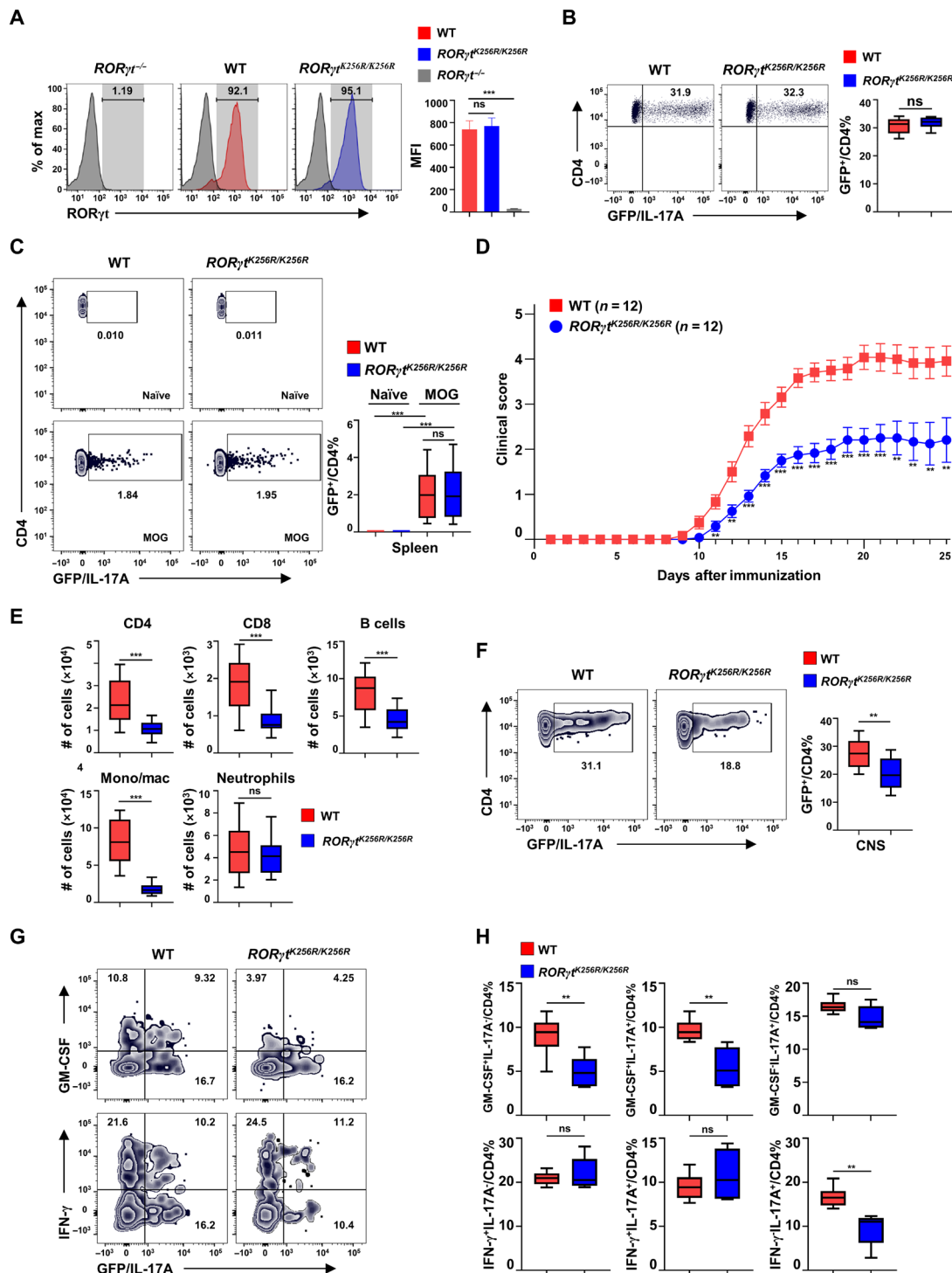


Fig. 4. ROR γ t^{K256R/K256R} mice have normal TH17 differentiation but develop impaired TH17-dependent EAE. (A) Flow cytometric analysis of ROR γ t in WT and ROR γ t^{K256R/K256R} CD4⁺ T cells polarized for 3 days under TH17 conditions (n = 4 to 6 per genotype). Bars are means \pm SEM. (B) Flow cytometric analysis of GFP⁺ (IL-17A⁺) cells among CD4⁺ T cells from indicated genotypes of cells differentiated under TH17 conditions as described in (A) (n = 4 per genotype). (C) Representative flow cytometric analysis of IL-17A⁺ cells among CD4⁺ T cells in the spleen of indicated genotype of mice either before (naïve) or 12 days after MOG₃₅₋₅₅ immunization (n = 4). (D) Mean clinical score of indicated mice different days after MOG₃₅₋₅₅ immunization. Bars are means \pm SEM. (E) Number of indicated immune cells recovered from the CNS of indicated EAE-induced mice from (D). (F) Representative flow cytometric analysis of GFP⁺ (IL-17A⁺) among CD4⁺ T cell infiltrate to the CNS of indicated mice shown in (D) (n = 10 per genotype). (G) Representative flow cytometric analysis of GM-CSF or IFN- γ expression in the lymphocyte infiltrated to the CNS of indicated mice shown in (D). (H) Quantification of GM-CSF⁺/CD4⁺ and IFN- γ ⁺/CD4⁺ cells in the CNS (n = 5 per genotype). **P < 0.01; ***P < 0.001 (two-tailed t test). (E, F, and H) Box plots: Median (central line), maximum, minimum (box ends), and outliers (extended lines).

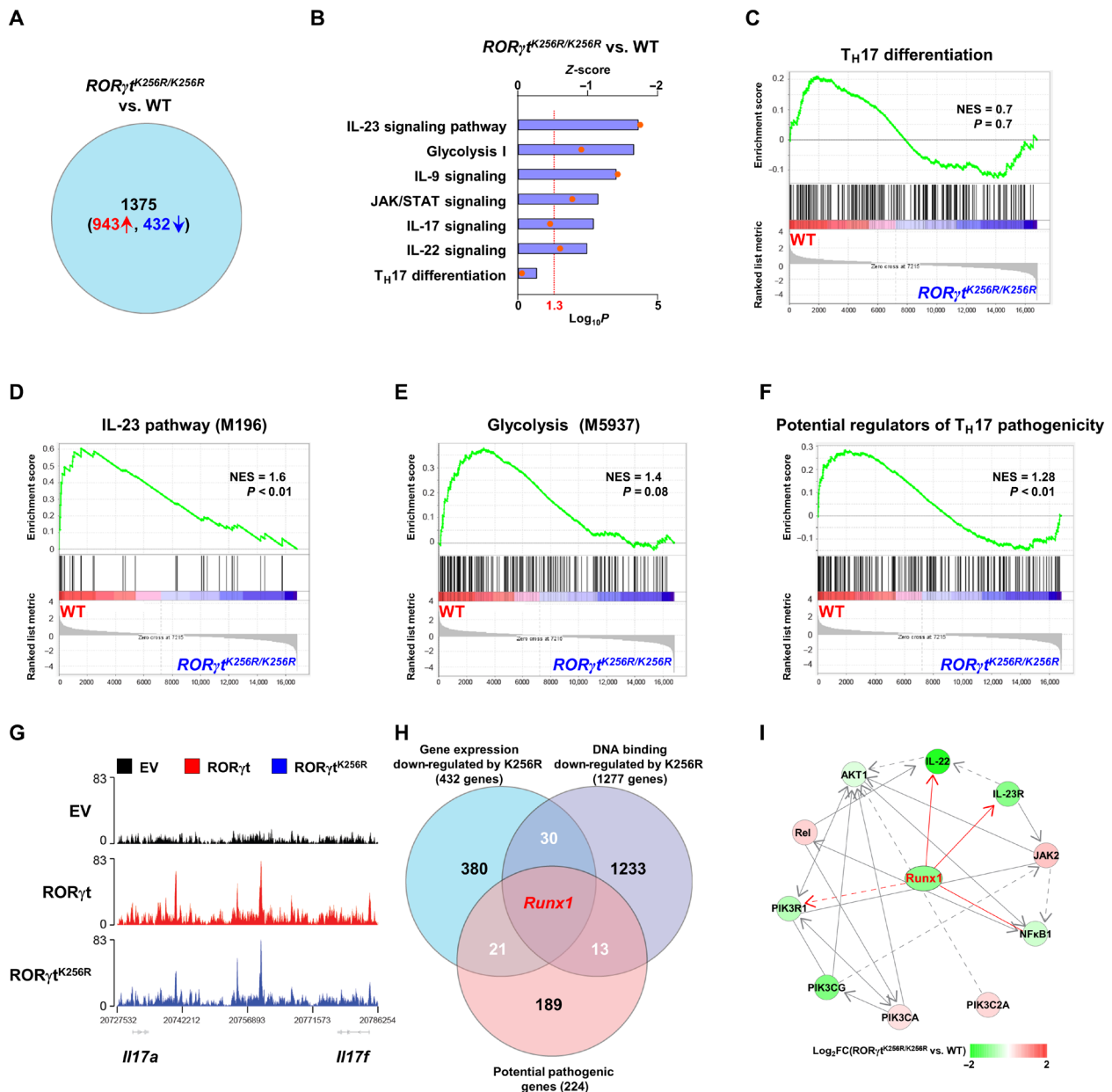


Fig. 5. ROR γ t-K256R impairs the pathways critical for the pathogenesis of EAE but not for the TH17 differentiation. (A to F) Computation analysis of RNA-seq data obtained from GFP⁺(IL-17A⁺) WT and ROR γ t^{K256R/K256R} CD4⁺ T cells polarized under TH17 conditions. (A) The number of DEGs (black) including up-regulated (red) and down-regulated (blue) genes with a cutoff at *P* value of <0.05 and FC > 1.5. (B) Ingenuity pathway analysis (IPA) canonical signaling pathway analysis of down-regulated pathways (*z*-score < 0) in ROR γ t^{K256R/K256R} versus WT TH17 cells. The dots denote *P* value, the vertical dotted line marks *P* value of 0.05 ($\log_{10}P = 1.3$). JAK, Janus kinase; STAT, signal transducer and activator of transcription. (C to F) GSEA plots showing the enrichment of genes critical for TH17 differentiation (C), IL-23 signaling (D), glycolysis (E), and pathogenesis of TH17-mediated EAE (F) between ROR γ t^{K256R/K256R} versus WT TH17 cells. NES, normalized enrichment score. Gene sets were derived from the molecular signaling database (MSigDB) for (C) to (E) and the previous report for (F) (35). (G) ChIP-seq analysis of ROR γ t DNA-binding peaks at the *Il17a* and *Il17f* loci in ROR γ t^{-/-} TH17 cells expressing indicated ROR γ t. (H) Venn diagram of gene overlapping among 432 down-regulated genes in ROR γ t^{K256R/K256R} TH17 cells, 1277 genes with decreased ROR γ t^{K256R}-binding activity, and 224 TH17 genes critical for the pathogenesis of EAE. (I) Protein-protein network analysis of differentially expressed regulators critical for the IL-23 signaling pathway between ROR γ t^{K256R/K256R} versus WT TH17 cells. Genes critical for IL-23 signaling pathway were customized on the basis of IPA-curated pathways.

(Fig. 5, B and E); both play important roles in the pathogenesis of EAE (33, 34). In addition, inflammatory cytokines such as IL-9, IL-17, and IL-22 signaling pathways were also down-regulated, likely reflecting a reduced ability to induce inflammation responsible for the tissue damages. We next subjected DEGs to gene set enrichment

analysis (GSEA) using the gene set specifically expressed in TH17 cells responsible for the development of pathogenic EAE (fig. S5D) (35) and found that ROR γ t^{K256R/K256R} TH17 cells had significantly reduced enrichment of the genes important for the pathogenesis of EAE when compared to the WT TH17 cells (Fig. 5F). This result is

consistent with the impaired EAE observed in $ROR\gamma^{\text{K256R/K256R}}$ mice. To determine the DEGs that are directly regulated by ROR γ , we performed chromatin immunoprecipitation sequencing (ChIP-seq) analysis to detect genome-wide ROR γ occupancy. ChIP-seq analysis in $T_{\text{H}}17$ cells revealed obvious ROR γ -binding peaks at *Il17a* and *Il17f* loci (Fig. 5G), consistent with published results (8, 36). ROR γ^{K256R} -binding peaks at the *Il17a* and *Il17f* loci were comparable to that of WT ROR γ , supporting that K256R did not affect ROR γ binding to *Il17* gene and thus its expression. Using our RNA-seq and ChIP-seq data, we cross-examined genes that were down-regulated in $ROR\gamma^{\text{K256R/K256R}}$ $T_{\text{H}}17$ cells with the genes that had reduced ROR γ^{K256R} -binding signals (see table S4 for the full list), identifying 31 genes (Fig. 5H). These 31 genes are considered directly regulated by ROR γ , and their reduced expression in $ROR\gamma^{\text{K256R/K256R}}$ $T_{\text{H}}17$ cells is likely due to reduced ROR γ^{K256R} binding and activating their expression. The 31 genes were then cross-examined with the gene set specifically expressed in $T_{\text{H}}17$ cells and responsible for the pathogenesis of EAE (fig. S5D) and identified *Runx1* (Fig. 5H). Furthermore, *Runx1* was found to be a core regulator for the IL-23 signaling pathway that was down-regulated in $ROR\gamma^{\text{K256R/K256R}}$ $T_{\text{H}}17$ cells by a protein-protein interaction network analysis (Fig. 5I), indicating that down-regulated IL-23 signaling pathway is likely due to down-regulated *Runx1* expression. Therefore, computational analysis of the transcriptome is consistent with the phenotypes observed in $ROR\gamma^{\text{K256R/K256R}}$ mice that ROR γ -K256R mutation does not affect $T_{\text{H}}17$ differentiation but impairs the effector function of $T_{\text{H}}17$ cells responsible for the development of EAE. *Runx1* is thus a possible ROR γ -regulated gene that is down-regulated in $ROR\gamma^{\text{K256R/K256R}}$ $T_{\text{H}}17$ cells and responsible for the observed defective effector function of $ROR\gamma^{\text{K256R/K256R}}$ $T_{\text{H}}17$ cells in the induction of EAE.

ROR γ -K256R mutation impairs ROR γ to bind and activate *Runx1* gene critical for EAE development

Our computational analysis identified *Runx1* as a potential gene responsible for impaired effector function of $ROR\gamma^{\text{K256R/K256R}}$ $T_{\text{H}}17$ cells, because (i) *Runx1* was down-regulated in $ROR\gamma^{\text{K256R/K256R}}$ / Tg^{Tcr2D2} $T_{\text{H}}17$ cells, which was also confirmed by individual quantitative polymerase chain reaction (qPCR) analysis (Fig. 6A); (ii) three prominent ROR γ -binding peaks close to the transcription start site of *Runx1* gene were identified by ChIP-seq analysis (Fig. 6B), and ROR γ^{K256R} binding signals at those peaks were substantially decreased (Fig. 6, B and C). ROR γ -binding sites were identified within the peak region by sequencing analysis (fig. S6A). Furthermore, individual ChIP assays also confirmed ROR γ binding to the peak region, whereas ROR γ^{K256R} -binding signals to this region were greatly decreased (Fig. 6D), which correlates to the decreased levels of *Runx1* mRNA in $ROR\gamma^{\text{K256R/K256R}}$ $T_{\text{H}}17$ cells (Fig. 6A); (iii) *Runx1* was a gene expressed in $T_{\text{H}}17$ cells and responsible for the pathogenesis of EAE, as deletion of *Runx1* impairs the EAE development (35, 37). To determine the effects of decreased ROR γ^{K256R} -binding peak signals on *Runx1* gene transcription, we cloned the DNA fragment covering the region with two potential ROR γ -binding sites (fig. S6A) to a luciferase reporter gene (pGL3) driven by a basic thymidine kinase (TK) promoter. The reporter activity was greatly stimulated by WT ROR γ but not as much by ROR γ^{K256R} (Fig. 6E). Therefore, reduced ROR γ^{K256R} binding to the *Runx1* gene correlates well with the reduced ability of ROR γ^{K256R} to stimulate *Runx1* gene expression. In contrast, WT ROR γ and ROR γ^{K256R} equivalently stimulated IL-17 promoter-luciferase report activity, which correlates with the equivalent

binding of ROR γ and ROR γ^{K256R} to and activation of the *Il17* gene (Figs. 4B and 5G). To further determine whether identified ROR γ -binding peaks are important for the expression of endogenous *Runx1* gene, the region containing the ROR γ -binding peaks was deleted using CRISPR-Cas9 with two guiding RNAs (Δ Rgn1) in $CD4^+$ T from mice expressing Cas9 (fig. S6A). At the same time, we used a non-targeting control (NTC) and a deleted adjacent region (Δ Rgn2) as negative controls, whereas deleted *Runx1* gene itself as a positive control (Δ Runx1) (fig. S6B for gating strategy for detecting *Runx1* expression after deletion). Deletion of the *Runx1* gene prevented *Runx1* expression [Fig. 6F and fig. S6C for mean fluorescence intensity (MFI) of *Runx1*], demonstrating a successful deletion strategy with CRISPR-Cas9. Furthermore, deletion of the ROR γ -binding region, but not the adjacent region or NTC, greatly reduced expression of *Runx1*, strongly indicating the critical function of the ROR γ -binding region in the stimulation of *Runx1* expression. Together with the results that ROR γ^{K256R} had decreased binding signals to *Runx1* (Fig. 6, B to D), these results suggest that reduced *Runx1* expression in $ROR\gamma^{\text{K256R/K256R}}$ $T_{\text{H}}17$ cells is due to impaired ROR γ^{K256R} -binding and activating *Runx1* gene expression. To determine whether the reduced level of *Runx1* in $ROR\gamma^{\text{K256R/K256R}}$ cells is responsible for the impaired development of EAE, we force-expressed *Runx1* in $ROR\gamma^{\text{K256R/K256R}}$ / Tg^{Tcr2D2} $CD4^+$ T cells (Fig. 6G) and adoptively transferred them to *Rag1*^{-/-} mice to induce EAE (Fig. 6H). Forced expression of *Runx1* significantly enhanced $ROR\gamma^{\text{K256R/K256R}}$ / Tg^{Tcr2D2} $CD4^+$ T cell function in the induction of EAE comparable to that of WT Tg^{Tcr2D2} T cells, which was also confirmed by increased infiltration of $CD4^+$ T cells to the CNS (Fig. 6I). Furthermore, the percentage of pathogenic IL-17⁺GM-CSF⁺ and IL-17⁺IFN- γ ⁺ cells in CNS was reduced in *Rag1*^{-/-} mice transferred with $ROR\gamma^{\text{K256R/K256R}}$ / Tg^{Tcr2D2} $CD4^+$ T cells compared to that transferred with WT Tg^{Tcr2D2} T cells but restored to WT levels in $ROR\gamma^{\text{K256R/K256R}}$ / Tg^{Tcr2D2} $CD4^+$ T cells expressing *Runx1* (Fig. 6J), consistent with rescuing the effector function of $ROR\gamma^{\text{K256R/K256R}}$ T cells by expressing *Runx1*. Collectively, our results demonstrated that the ubiquitination site of ROR γ -K256, which is not essential for $T_{\text{H}}17$ differentiation, regulates the effector function of $T_{\text{H}}17$ cells required for inducing EAE via up-regulating *Runx1*. By decoupling the function of ROR γ in the differentiation and effector function of $T_{\text{H}}17$ cells, we demonstrated that ROR γ also regulates the effector function of $T_{\text{H}}17$ cells.

DISCUSSION

The transcription factor ROR γ , which is encoded by gene *Rorc*, is well known for instructing the differentiation of $T_{\text{H}}17$ cells. Activation of naïve T cells in the presence of TGF- β , IL-6, and/or IL-23 is sufficient to up-regulate ROR γ , which instructs the differentiation into $T_{\text{H}}17$ cells (14, 38–41). The hallmark of $T_{\text{H}}17$ differentiation is the activation and expression of IL-17. ROR γ directly binds to *Il17a* and *Il17f* gene loci to stimulate their expression (8, 36), which explains the essential function of ROR γ in the differentiation of IL-17-producing $T_{\text{H}}17$ cells. Differentiated $T_{\text{H}}17$ cells are able to induce tissue inflammation involved in the pathogenesis of autoimmune diseases, including psoriasis, inflammatory bowel disease, and multiple sclerosis (2, 3, 14). Previous studies using *ROR\gamma*^{-/-} mice demonstrated the essential function of ROR γ in $T_{\text{H}}17$ cell-mediated autoimmunity such as EAE (7, 9). *ROR\gamma*^{-/-} mice are resistant to EAE and other $T_{\text{H}}17$ -mediated autoimmunity, which is due to the lack of ROR γ -dependent generation of $T_{\text{H}}17$ cells. The question remains

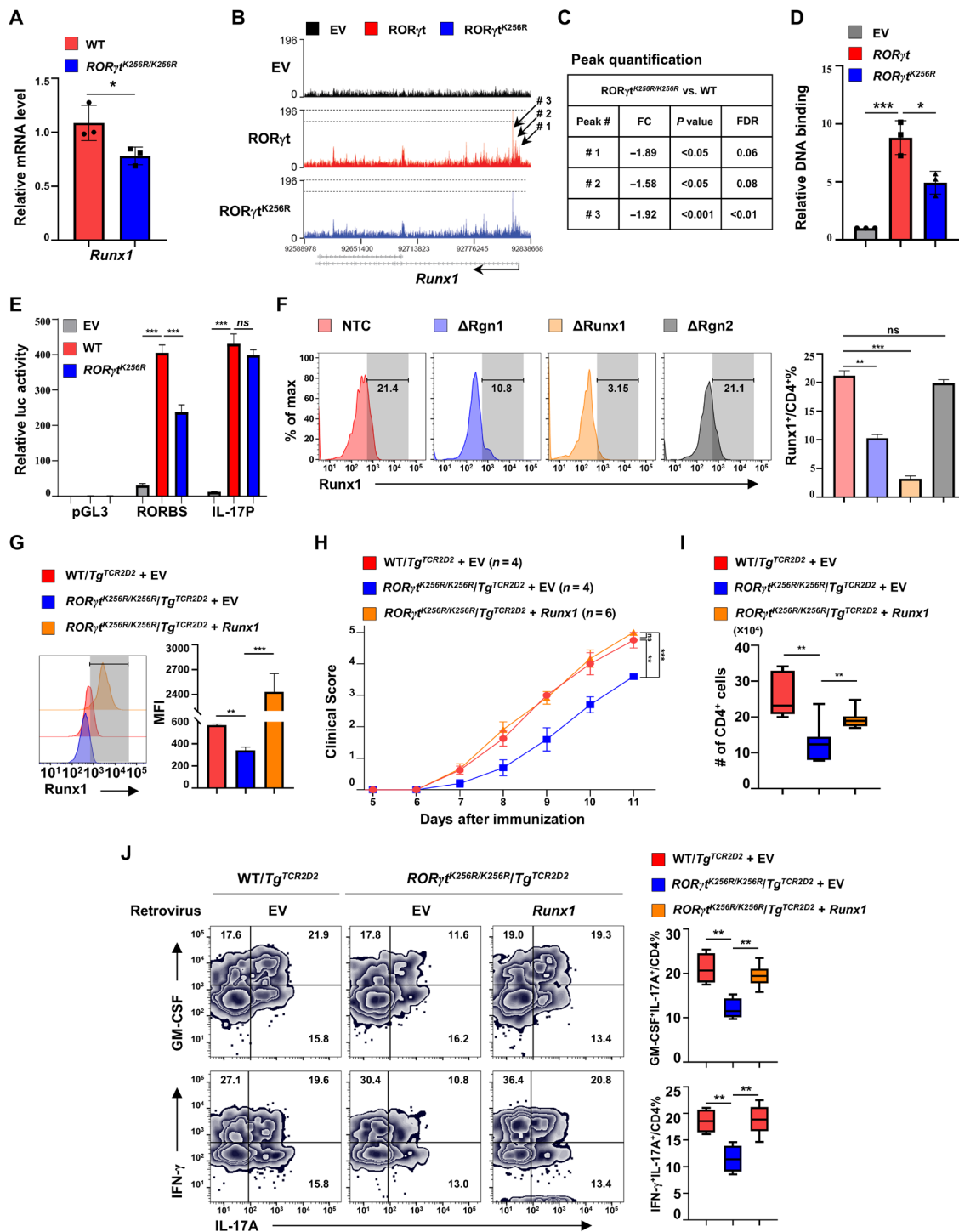


Fig. 6. ROR γ t-K256R mutation impairs ROR γ t to bind and activate *Runx1* gene critical for EAE development. (A) qPCR of *Runx1* mRNA in indicated genotypes of T_H17 cells (n = 3). Bars are means \pm SEM. (B) ChIP-seq analysis of ROR γ t DNA-binding peaks at the *Runx1* locus in ROR γ ^{K256R/K256R} T_H17 cells transduced with indicated vectors. (C) FC of ROR γ t-binding peaks indicated in (B). FDR, false discovery rate. (D) ChIP analysis of ROR γ t-binding signals at the *Runx1* gene locus shown in (B) (n = 3). Bars are means \pm SEM. (E) Luciferase (luc) activity from HEK 293T cells transfected with a pGL3 basic luciferase vector, a luciferase reporter with ROR γ t-binding sites (RORBS)-containing DNA fragment cloned from *Runx1* locus and a basic TK promoter or IL-17 promoter (IL-17P) luciferase reporter under indicated conditions. (F) Flow cytometric analysis of Runx1 among GFP⁺(Cas9⁺) T_H17 cells retrovirally transduced with indicated single-guide RNAs (n = 4). (G) Flow cytometric analysis of Runx1 among indicated genotypes of T_H17 cells retrovirally transduced with GFP alone (EV) or together with Runx1 (n = 3). (H) Mean clinical score of *Rag1*^{-/-} mice adoptively transferred with indicated genotypes of T_H17 cells expressing GFP alone or with Runx1 shown in (G). (I) Number of infiltrating CD4⁺ T cells recovered from the CNS of the EAE-induced mice shown in (H). (J) Flow cytometric analysis of GM-CSF, IFN- γ , and IL-17A in CD4⁺ T cells recovered from the CNS of EAE-induced mice shown in (H). *P < 0.05; **P < 0.01; ***P < 0.001 (two-tailed t test). (I and J) Box plot: Median (central line), maximum, minimum (box ends), and outliers (extended lines).

whether ROR γ t plays a role in the effector function of T_H17 cells involved in autoimmunity. The mutation ROR γ t^{K256R} we identified at the ubiquitination site does not affect the generation of IL-17-producing T_H17 cells but impairs the effector function of T_H17 cells in the induction of EAE. Further, ROR γ t^{K256R/K256R} mice have normal T_H17 differentiation both in vitro and in vivo but have greatly impaired T_H17 immune responses that led to EAE. In addition, ROR γ t^{K256R/K256R} mice have normal ROR γ t-dependent thymocyte development and lymph node genesis including Peyer's patches. This study thus reveals a previously unidentified and essential ROR γ t function in effector T_H17 cells in addition to T_H17 differentiation. This study informs the development of ROR γ t-based therapies that specifically target the effector function of T_H17 cells responsible for the pathogenesis of autoimmunity. This will have a significant impact on the clinical treatment of T_H17-mediated autoimmunity, as usually medical treatment is performed after a diagnosis of autoimmune diseases resulting from the effector function of already developed T_H17 cells.

Runx1 is a transcription factor known to regulate hematopoiesis (42–44) and oncogenesis (45). *Runx1* was reported to play a role in T_H17 cells. Particularly relevant to T_H17 cells involved in the pathogenesis of EAE, *Runx1* has been shown to be up-regulated in T_H17 cells and work together with T-bet to stimulate IFN- γ expression that is believed to be responsible for the induction of EAE (37). However, it is not clear how *Runx1* is up-regulated in T_H17 cells. Our results demonstrate that *Runx1* expression is stimulated by ROR γ t, as ROR γ t-binding peaks were detected by ChIP-seq on the promoter region of the *Runx1* gene. The conserved ROR γ t-binding sequence was identified within the detected peaks, and the deletion of the DNA fragment containing the ROR γ t-binding site greatly reduced *Runx1* expression. Moreover, ROR γ t^{K256R} has reduced binding signals at the ROR γ t-binding region, which correlates with the impaired ability of ROR γ t^{K256R} in stimulating a luciferase reporter gene driven by the ROR γ t-binding region identified in the *Runx1* locus. These results demonstrate the mechanisms for how ROR γ t regulates the effector function of T_H17 cells via up-regulating *Runx1* expression and why ROR γ t-K256R mutation impairs the effector function of T_H17 cells. An in vitro study showed that *Runx1* can stimulate ROR γ t expression, which is, however, inhibited by T-bet (46). Because T-bet is required for IFN- γ expression, this seems to suggest that T-bet and ROR γ t inhibit each other. In addition, *Runx1* has been shown to be required for forkhead box P3 (Foxp3) expression (47), and *Runx1* is able to stimulate itself expression via an autoregulation mechanism (48). The function of *Runx1* is thus complicated and dependent on the microenvironment. In our study, both in vitro and in vivo, we did not find obvious changes in the levels of ROR γ t expression in ROR γ t^{K256R/K256R} T_H17 cells that have lower levels of *Runx1*, thus not supporting the role of K256 ubiquitination in the regulation of ROR γ t and T_H17 differentiation. Furthermore, even forced expression of *Runx1* in ROR γ t^{K256R/K256R} CD4⁺ T cells does not affect T_H17 differentiation. Therefore, ROR γ t-regulated *Runx1* expression does not affect T_H17 differentiation but is required for the effector function of T_H17 cells that mediate pathogenic EAE.

Dysregulated T_H17 cells are often associated with autoimmune diseases such as EAE and psoriasis resulting from a reaction to self-antigens (49). In addition to IL-17, IL-23 also plays an important role in T_H17 cell-dependent autoimmune diseases (1, 50) such as EAE and psoriasis (51, 52). Neutralizing antibodies for IL-23 and IL-17 or their receptors are used for the treatment of these autoimmune conditions (53–55). Therefore, inhibiting the T_H17 pathway is effective

for treating autoimmune conditions (52, 56). Our results show that the IL-23 signaling pathway is down-regulated in ROR γ t^{K256R/K256R} T_H17 cells. Further, network analysis supports that *Runx1* is a core regulator for the IL-23 pathway and down-regulated IL-23 pathway thus likely resulting from down-regulated *Runx1*. Therefore, ROR γ t-regulated *Runx1* seems to control the effector function of T_H17 cells at least partially through regulating the IL-23 pathway known to be critical for T_H17-mediated autoimmunity.

Considering the essential function of ROR γ t in T_H17 cells, ROR γ t inhibitors are being developed to treat T_H17-dependent autoimmunity (5, 11–13, 57). However, these ROR γ t inhibitors mostly target T_H17 differentiation and ROR γ t-dependent thymocyte development. Inhibition of ROR γ t-dependent thymocyte development leads to a high frequency of thymic lymphoma (8, 58, 59). Our results demonstrate that a posttranslational ubiquitination event can dictate ROR γ t function in T_H17-dependent responses involved in autoimmunity. However, this ubiquitination event is dispensable for thymocyte development and T_H17 differentiation. Therefore, targeting this ROR γ t ubiquitination event is a potential treatment for T_H17-dependent autoimmune disease without induction of thymocyte lymphoma. Currently, it remains unknown about the ROR γ t ubiquitination pathway including the enzymes involved in the ubiquitination of ROR γ t at K256. Illustrating the detailed mechanisms responsible for ubiquitination of ROR γ t will facilitate the development of novel treatments that target the ROR γ t-dependent effector function of T_H17 cells responsible for autoimmunity while minimizing the other toxic effects such as lymphoma.

MATERIALS AND METHODS

Experimental design

The objective of this study was to determine whether ROR γ t plays a role in effector function of T_H17 cells in addition to its known function in T_H17 differentiation. To achieve this goal, we dissected the function of ROR γ t with K-R mutations in thymus development, T_H17 differentiation, and induction of EAE. ROR γ t-K256R mutation did not affect T_H17 differentiation but impaired the effector function of T_H17 cells responsible for inducing EAE, which was also confirmed by the in vivo studies using ROR γ t^{K256R/K256R} mice. RNA-seq and ChIP-seq assays identified *Runx1* as a direct target of ROR γ t in the regulation of effector function of T_H17 cells.

Mice

All male and female mice used for experiments were between 6 and 12 weeks old; age-matched littermates were used. The ROR γ t^{-/-} (*Rorc*^{tm1Litt}, stock no. 007571) mouse strain was described previously (18). The ROR γ t^{K256R/K256R} point-mutated mice were designed and generated by Biocytogen LLC. *Rag1*^{-/-} (*Rag1*^{tm1Mom}, stock no. 002216), *Tg*^{Tcr2D2} (*Tcra*2D2 and *Tcrb*2D2, stock no. 006912), IL-17A-GFP (*Il17a*^{tm1Bgen}, stock no. 018472), CRISPR-Cas9-enhanced GFP (EGFP) [*Gt(ROSA)26Sor*^{em1.1(CAG-cas9₆-EGFP)Rsky}, stock no. 028555], and C57BL/6J (stock no. 000664) mice were purchased from the Jackson Laboratory. For some assays, the mice were crossed to generate ROR γ t^{-/-}/*Tg*^{Tcr2D2}, ROR γ t^{K256R/K256R}/*Tg*^{Tcr2D2}, and ROR γ t^{K256R/K256R}/*IL-17A-IRES-GFP-KI* mice. All mice were bred at the C57BL/6J background and maintained in a pathogen-free animal facility at City of Hope. All animal experiments were conducted per the protocols approved by the Institutional Animal Care and Use Committee at City of Hope. Statistical tests were not used to predetermine sample

sizes. The sample sizes were chosen on the basis of previous studies of our own and by others in the field (8). The sample sizes are indicated in the figure legends or figures. Allocation of mice to experimental groups was random.

Induction and assessment of EAE

Active EAE was induced and assessed as previously described (8). Briefly, mice were immunized with 200 mg of MOG_{35–55} (Hooke Laboratories) in complete Freund's adjuvant by subcutaneous injection at two dorsal sites at day 0, followed by two intraperitoneal injections of 80 ng of pertussis toxin at days 0 and 1. For passive EAE, *Rag1*^{−/−} mice were adoptively transferred with 1×10^5 TCR^{MOG}-expressing (*Tg*^{Tcr2D2}) T_H17 cells that were differentiated under T_H17 polarization condition, followed by an immunization with MOG at 7 days after injection. In certain experiments, WT RORγt cells were virally transduced with retrovirus expressing GFP alone, while RORγt^{K256R/K256R} cells were transduced with retrovirus expressing Runx1 and GFP before in vitro T_H17 differentiation. In other experiments, RORγt^{−/−} cells were transduced with an empty vector or vectors encoding WT RORγt, RORγt^{K87R}, RORγt^{K99R}, RORγt^{K256R}, or RORγt^{K288R}. All transduced cells were sorted for CD4 and GFP expression before adoptive transfer into mice. Severity of EAE was monitored, and a clinical score of 0 to 5 was assigned (30): 0 = no disease, 0.5 = partially limp tail, 1 = paralyzed tail, 2 = hindlimb weakness, 3 = hindlimb paralysis, 4 = hindlimb and forelimb paralysis, and 5 = moribundity and death.

Isolation of naïve CD4⁺ T cell isolation and in vitro T_H17 differentiation

Murine CD4⁺ T cells were isolated from spleens by negative selection using the Naive CD4⁺ T Cell Isolation Kit (Miltenyi Biotec). Suspensions of 4×10^5 cells/ml in RPMI 1640 medium (Corning Inc.) containing 2 mM L-glutamine, 50 μM β-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% fetal bovine serum (FBS) were activated with hamster anti-CD3 (0.25 μg/ml; 145-2C11, BioLegend) and hamster anti-CD28 (1 μg/ml; 37.51, BioLegend) antibodies overnight in 24-well plates precoated with rabbit anti-hamster immunoglobulin G fraction (0.1 mg/ml; catalog no. 55398, MP Biomedicals). The following T_H17 differentiation was carried out by supplementing to the culture medium mentioned above with TGF-β (2 ng/ml; Miltenyi Biotec), IL-6 (20 ng/ml; Miltenyi Biotec), anti-IL-4 (2 μg/ml; 11B11, BioLegend), and anti-IFN-γ (2 μg/ml; XMG 1.2, BioLegend), and additional IL-23 (20 ng/ml; Miltenyi Biotec) was also added for the induction of pathogenic T_H17 cells.

Retroviral transduction

The retroviral vector murine stem cell virus (MSCV)–internal ribosomal entry site (IRES)–GFP (MIGR1, a gift from W.S. Pear, University of Pennsylvania) was used to clone WT or mutated RORγt. MSCV vector for expressing Runx1 was a gift from I. Taniuchi (RIKEN Center for Integrative Medical Sciences, Japan). Vectors were first transfected to Platinum-E (Cell Biolabs) retroviral packaging cells using BioT transfection reagent (Bioland Scientific), followed by a change of fresh medium at 24 hours. The virus-containing supernatant was collected at 48 and 72 hours, filtered with a 0.45-μm polyvinylidene difluoride (PVDF) syringe filter (Millipore), and used to transduce T cells or stored for future use at −80°C. Transduction of activated CD4⁺ T was performed by spin infection with viral supernatants (two, 500g, 30°C for 2 hours) in the presence of polybrene

(8 μg/ml; Sigma-Aldrich). After spinning, the plates were incubated at 37°C for 3 hours. The viral supernatant was replaced with fresh culture medium with polarizing cytokines for in vitro differentiation.

CRISPR-Cas9-mediated genomic DNA deletion

Single-guide RNA (sgRNA) of *Runx1*, targeting the exon region (Addgene, library 67988), was cloned to pMSCV-U6sgRNA(Bbs I)-PGKpuro2ABFP (Addgene, 102796) with modification of Bbs I sites (table S1) for using universal primer design through this study. To generate plasmids for the deletion of large fragments of genomic DNA, PCR products of two U6 promoter–sgRNA cassettes and a phosphoglycerate kinase (PGK) promoter–TagBFP cassette were assembled using the Golden Gate assembly method and inserted into the MIGR1 vector with disrupted Bsp MI site. Bbs I sites and newly introduced Bsp MI sites were used for the insertion of gRNAs into each cassette. sgRNAs were delivered to the cells by retroviral transduction. The U6 promoter–driven transcription of sgRNAs in each cassette was confirmed by examining sgRORγt expression in T_H17 cells together with a simultaneously expressed NTC sgRNA in another cassette. Three sgRNAs (sgRNA1, sgRNA2, and sgRNA3; fig. S6A) targeting the sequence of the *Runx1* gene were designed by using an online tool (CRISPOR, <http://crispor.tefor.net/>). A simultaneous expression of sgRNA1 with sgRNA2 or sgRNA2 with sgRNA3 in Cas9-expressing cells was designed for deletion of the RORγt binding region (Rgn1) and the adjacent region (Rgn2) without RORγt binding site. sgRNA primers are listed in table S1.

In vitro T cell–development assay

Murine thymocytes from RORγt^{−/−} mice were subjected to fluorescence-activated cell sorting for isolating DN (Thy1.2⁺CD4[−]CD8[−]) cells. Sorted cells at 5×10^5 cells/ml were cultured overnight on an 80% confluent bone marrow-derived stromal cell line (OP9) expressing the delta like canonical Notch ligand 4 (Dll4/DL4) (OP9-DL4) monolayer (a gift from E.V. Rothenberg) in 24-well culture plates with α-modified minimum essential medium (Invitrogen Life Technologies) supplemented with 20% FBS, penicillin-streptomycin (100 U/ml), 2 mM L-glutamine (Invitrogen Life Technologies), and recombinant mouse IL-7 (5 ng/ml; PeproTech). The cells were then transduced with RORγt carrying K/R mutations as described above. Cocultures were maintained for an additional 3 days in the fresh medium containing murine IL-7 (5 ng/ml). Cells were harvested for flow cytometry analysis.

Apoptosis assays

Murine thymocytes were collected by smashing the thymus in a 40-μm cell strainer. Cells were suspended in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 2 mM L-glutamine at 1×10^6 cells/ml and cultured for 0, 3, 6, 12, 18, or 24 hours. Thymocytes were then incubated with anti-Thy1.2 antibody and a fixable LIVE/DEAD near-infrared dye (Thermo Fisher Scientific). After two washes, the cells were stained with 5 μl of phycoerythrin-annexin V in 100 μl of binding buffer containing 0.01 M Hepes (pH 7.4), 0.14 M NaCl, and 2.5 mM CaCl₂ for 15 min. An additional 400 μl of binding buffer was added to the suspension before analysis.

RNA-seq and analysis

CD4⁺ T cells isolated from RORγt^{WT}/IL-17A-GFP^{+/-} and RORγt^{K256R/K256R}/IL-17A-GFP^{+/-} mice were differentiated into pathogenic T_H17 cells as described above. RNA was extracted from sorted $\sim 1 \times 10^6$ GFP-expressing T_H17 cells (CD4⁺GFP⁺) using an RNAeasy mini kit

(QIAGEN). Each group contained three replicates from different mice. Quality control, library preparation, and sequencing were performed at Novogene. The analysis was performed through Partek Flow. Briefly, the sequence reads were aligned to the mouse whole genome (GRCm38) with validation of quality through prealignment and postalignment quality assurance/quality control (QA/QC). Aligned reads were further subjected to quantification using the Partek expectation/maximization (E/M) algorithm and normalization to counts per million with 0.001 added to each. The identification of differentially expressed features was performed through the Partek gene specific analysis (GSA) algorithm that applies multiple statistical models to each gene. Genes with total counts over 30 were considered to be statistically expressed in the cells. The expression values of pathogenic genes were extracted and subjected to ingenuity pathway analysis (IPA), gene set enrichment assay (GSEA), and network analysis.

Chromatin immunoprecipitation sequencing

In vitro-activated (see above) *RORγt*^{-/-} CD4⁺ T cells that were transduced with retroviruses carrying GFP, *RORγt*-3xFlag/GFP, or *RORγt*^{K256R}-3xFlag/GFP were used. After T_H17 polarization, 2 × 10⁷ cells were fixed in 1% formaldehyde at room temperature for 10 min to cross-link proteins with chromatin. The reaction was stopped with incubation in glycine for 5 min. Genomic DNA was fragmented with enzyme cocktail (ChIP-IT Express Enzymatic kit, Active Motif) for 10 min as directed. Cell lysates were centrifuged at 15,000 rpm for 10 min to remove debris, and the supernatant was used for immunoprecipitation. An equal amount of DNA was incubated with anti-FLAG (M2, Sigma-Aldrich) overnight, followed by precipitation with protein G agarose beads. Beads complexed with DNA fragments were extensively washed five times, and DNA was eluted, followed by reverse cross-linking. Recovered DNA was subjected to NovaSeq with 51-base pair (bp) paired-end sequencing length. Primers used in reverse-transcription quantitative PCR (RT-qPCR) are listed in table S1. Reads were analyzed using Partek Flow through alignment to the mm10 mouse genome using the Burrow-Wheeler aligner (BWA). Peaks were identified with the model-based analysis of ChIP-seq 2 (MACS2) tool (version 2.1.1) and quantified with a minimum region size of 50 bp.

Flow cytometry

For surface staining, cells isolated from mice or in vitro culture were directly stained with antibodies in phosphate-buffered saline (PBS) with 2% FBS and 1 mM EDTA at 4°C for 15 min. A blocking of Fc receptors with anti-CD16/32 antibody was carried out in case monocytes/macrophages were present. For staining transcription factors, cells were prestained for surface markers, fixed, and permeabilized in TF Fix/Perm buffer (BD Biosciences) at 4°C for 20 min and washed once with TF Perm/Wash buffer. Cells were stained for target proteins (see antibody list below) in the TF Perm/Wash buffer at 4°C for 15 min. For cytokine staining, cells were prestimulated with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma-Aldrich) and ionomycin (750 ng/ml; Sigma-Aldrich) for 3 hours ahead of staining. Meanwhile, GolgiStop (BD Biosciences) was cotreated to block protein transport. In certain experiments, cells were stained with surface markers and/or fixable live/dead dye (Thermo Fisher Scientific). Cells were fixed and permeabilized with CytoFix/CytoPerm buffer (BD Biosciences), followed by staining for cytokines in the Perm/Wash buffer (BD Biosciences) after washing. To measure cell proliferation, either naïve CD4⁺ T cells or in vitro-differentiated T_H17 cells for adoptive

transfer were stained with CellTrace Violet dye (Thermo Fisher Scientific) in PBS (1:5000) at room temperature for 20 min. After washing, naïve CD4⁺ T cells were subjected to anti-CD3/anti-CD28 stimulation and T_H17 differentiation for measuring in vitro proliferation, and T_H17 cells were sorted out and injected to Rag1^{-/-} mice for measuring hemostatic proliferation at day 3. Subsequent analysis was performed in the BD LSRFortessa flow cytometer.

The following antibodies were used for flow cytometric assay: anti-CD45 (BioLegend, clone 30-F11), anti-CD3 (BioLegend, 145-2C11), CD4 (BioLegend, RM4-5), anti-CD8 (BioLegend, 53-6.7), anti-CD19 (BioLegend, 1D3), anti-lymphocyte antigen 6 complex locus G6D (Ly6G) (BioLegend, 1A8), anti-CD62L (BioLegend, MEL-14), anti-CD44 (BioLegend, IM-7), anti-IFN-γ (BioLegend, XMG-1.2), anti-GM-CSF (BioLegend, MP1-22F9), killer cell lectin-like receptor subfamily B member 1C (Klrb1c/NK1.1) (BioLegend, PK136), anti-CD11b (eBioscience, M1/70), anti-Ly6C (eBioscience, HK1.4), anti-Thy1.2 (eBioscience, 53-1.2), anti-IL-17A (eBioscience, eBio17B7), anti-Runx1 (eBioscience, RXDMC), anti-IL-22 (eBioscience, 1H8PWSR), anti-Foxp3 (eBioscience, FJK-16 s), anti-RORγt (BD Biosciences, Q31-378), and CD1d-tetramer [National Institutes of Health (NIH), PBS-57].

Western blotting

A total 1.5 × 10⁷ T_H17 cells were lysed in radioimmunoprecipitation assay buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, and leupeptin (1 μg/ml). Ubiquitinated proteins were precipitated and enriched with 20 μl of equilibrated agarose-Tandem Ubiquitin Binding Entities 2 (TUBE2) (Lifesensors) at 4°C for 4 hours. Agarose-TUBE2-protein complex were washed with Tris buffered saline containing 0.1% Tween-20 (TBST) and subjected to heating in 2× Laemmli sample buffer (Bio-Rad) with β-mercaptoethanol at 90°C for 5 min. The supernatant containing precipitated proteins was subjected to SDS-polyacrylamide gel electrophoresis, and the protein was transferred to the PVDF membrane. Target proteins were sequentially immunoblotted with relevant primary antibodies and fluorescent secondary antibodies (LI-COR Biosciences), followed by measuring fluorescent intensity with a LI-COR Odyssey blot imager (LI-COR Biosciences). The eventual samples for Western blotting were pooled from three different experiments. Quantification of ubiquitination signals of blots showing in Fig. 2 (E and F) was performed to the area above 50 kDa.

Reverse-transcription quantitative PCR

Total RNA was extracted using the RNeasy mini kit (QIAGEN) as directed. A Tetro complementary DNA synthesis kit (Bioline) was used for reverse transcription. Subsequent qPCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems) in the QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific). The primers used for qPCR are listed in table S1. The amplification efficiency of all primers was tested and optimized. Gene expression was calculated with the delta-delta Ct (ΔΔCt) method normalized to the control gene encoding β-actin and glyceraldehyde-3-phosphate dehydrogenase, and all measurements were performed in triplicate.

Luciferase assay

Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM glutamine,

penicillin (100 U/ml), and streptomycin (100 mg/ml). A total of 8×10^5 cells were seeded to each well of a six-well plate and transfected with the reporter vectors (400 ng), pSV40-*Renilla* luciferase vector (200 ng), and expression vectors (2 μ g) using BioT transfection reagent (Bioland Scientific, Paramount, CA). The same amount of plasmid DNA was used by adjusting with an empty vector. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) per the manufacturer's instruction and normalized against *Renilla* luciferase activities. "Relative luciferase activities" were plotted with further normalization of luciferase activities of each group to the pGL3-basic reporter vector plus the empty vector group. The generation of reporter plasmids was done by PCR amplification of *Runx1* genomic DNA containing the ROR γ t-binding region and a subsequent insertion upstream of a mini TK promoter that was cloned to pGL3-basic vector (Promega) for a minimal expression of luciferase. Cloning primers and mini TK promoter sequence are listed in table S1.

Statistical analysis

The ratio of rescue for thymocyte development and T_H17 differentiation in Fig. 2 (A and B) was calculated as relative ratio of the extent of ROR γ t^{-/-} cells transduced with ROR γ t mutants to that of cells transduced with WT ROR γ t. The statistical parameters are indicated in the figure legends. The results were analyzed for statistical significance with unpaired Student's *t* test. Bodyweights are presented as means \pm SD, and other data are shown as means \pm SEM. *P* values are calculated using GraphPad Prism and presented where the statistical significance (*P* < 0.05) was found.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <https://science.org/doi/10.1126/sciadv.adc9221>

[View/request a protocol for this paper from Bio-protocol.](#)

REFERENCES AND NOTES

1. Y. Lee, A. Awasthi, N. Yosef, F. J. Quintana, S. Xiao, A. Peters, C. Wu, M. Kleinewietfeld, S. Kunder, D. A. Hafler, R. A. Sobel, A. Regev, V. K. Kuchroo, Induction and molecular signature of pathogenic T_H17 cells. *Nat. Immunol.* **13**, 991–999 (2012).
2. L. Codarri, G. Gyulveszi, V. Tosevski, L. Hesse, A. Fontana, L. Magnenat, T. Suter, B. Becher, ROR γ t drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat. Immunol.* **12**, 560–567 (2011).
3. M. El-Behi, B. Ciric, H. Dai, Y. Yan, M. Cullimore, F. Safavi, G. X. Zhang, B. N. Dittel, A. Rostami, The encephalitogenicity of T_H17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat. Immunol.* **12**, 568–575 (2011).
4. M. M. Elloso, M. Gomez-Angelats, A. M. Fourie, Targeting the Th17 pathway in psoriasis. *J. Leukoc. Biol.* **92**, 1187–1197 (2012).
5. J. Skepner, R. Ramesh, M. Trocha, D. Schmidt, E. Baloglu, M. Lobera, T. Carlson, J. Hill, L. A. Orband-Miller, A. Barnes, M. Boudjelal, M. Sundrud, S. Ghosh, J. Yang, Pharmacologic inhibition of ROR γ t regulates Th17 signature gene expression and suppresses cutaneous inflammation in vivo. *J. Immunol.* **192**, 2564–2575 (2014).
6. G. B. Choi, Y. S. Yim, H. Wong, S. Kim, H. Kim, S. V. Kim, C. A. Hoeffler, D. R. Littman, J. R. Huh, The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. *Science* **351**, 933–939 (2016).
7. I. I. Ivanov, B. S. McKenzie, L. Zhou, C. E. Tadokoro, A. Lepelletier, J. J. Lafaille, D. J. Cua, D. R. Littman, The orphan nuclear receptor ROR γ t directs the differentiation program of proinflammatory IL-17⁺ T helper cells. *Cell* **126**, 1121–1133 (2006).
8. Z. He, J. Ma, R. Wang, J. Zhang, Z. Huang, F. Wang, S. Sen, E. V. Rothenberg, Z. Sun, A two-amino-acid substitution in the transcription factor ROR γ t disrupts its function in T_H17 differentiation but not in thymocyte development. *Nat. Immunol.* **18**, 1128–1138 (2017).
9. Z. He, J. Zhang, Z. Huang, Q. Du, N. Li, Q. Zhang, Y. Chen, Z. Sun, Sumoylation of ROR γ t regulates T_H17 differentiation and thymocyte development. *Nat. Commun.* **9**, 4870 (2018).
10. S. Okada, J. G. Markle, E. K. Deenick, F. Mele, D. Averbuch, M. Lagos, M. Alzahrani, S. Al-Muhsen, R. Halwani, C. S. Ma, N. Wong, C. Soudais, L. A. Henderson, H. Marzouqa, J. Shamma, M. Gonzalez, R. Martinez-Barricarte, C. Okada, D. T. Avery, D. Latorre, C. Deswarte, F. Jabot-Hanin, E. Torrado, J. Fountain, A. Belkadi, Y. Itan, B. Boisson, M. Migaud, C. S. Arlehamn, A. Sette, S. Breton, J. McCluskey, J. Rossjohn, J. P. de Villartay, D. Moshous, S. Hambleton, S. Latour, P. D. Arkwright, C. Picard, O. Lantz, D. Engelhard, M. Kobayashi, L. Abel, A. M. Cooper, L. D. Notarangelo, S. Boisson-Dupuis, A. Puel, F. Sallusto, J. Bustamante, S. G. Tangye, J. L. Casanova, Impairment of immunity to *Candida* and *Mycobacterium* in humans with bi-allelic RORC mutations. *Science* **349**, 606–613 (2015).
11. S. Xiao, N. Yosef, J. Yang, Y. Wang, L. Zhou, C. Zhu, C. Wu, E. Baloglu, D. Schmidt, R. Ramesh, M. Lobera, M. S. Sundrud, P. Y. Tsai, Z. Xiang, J. Wang, Y. Xu, X. Lin, K. Retschmer, P. B. Rahl, R. A. Young, Z. Zhong, D. A. Hafler, A. Regev, S. Ghosh, A. Marson, V. K. Kuchroo, Small-molecule ROR γ t antagonists inhibit T helper 17 cell transcriptional network by divergent mechanisms. *Immunity* **40**, 477–489 (2014).
12. Z. Huang, H. Xie, R. Wang, Z. Sun, Retinoid-related orphan receptor γ t is a potential therapeutic target for controlling inflammatory autoimmunity. *Expert Opin. Ther. Targets* **11**, 737–743 (2007).
13. J. R. Huh, D. R. Littman, Small molecule inhibitors of ROR γ t: Targeting Th17 cells and other applications. *Eur. J. Immunol.* **42**, 2232–2237 (2012).
14. S. L. Gaffen, R. Jain, A. V. Garg, D. J. Cua, The IL-23-IL-17 immune axis: From mechanisms to therapeutic testing. *Nat. Rev. Immunol.* **14**, 585–600 (2014).
15. J. S. Bezbradica, T. Hill, A. K. Stanic, L. Van Kaer, S. Joyce, Commitment toward the natural T (iNKT) cell lineage occurs at the CD4⁺8⁺ stage of thymic ontogeny. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 5114–5119 (2005).
16. A. V. Rachtitskaya, A. M. Hansen, R. Horai, Z. Li, R. Villasmil, D. Luger, R. B. Nussenblatt, R. R. Caspi, Cutting edge: NKT cells constitutively express IL-23 receptor and ROR γ t and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion. *J. Immunol.* **180**, 5167–5171 (2008).
17. T. Egawa, G. Eberl, I. Taniuchi, K. Benlagha, F. Geissmann, L. Hennighausen, A. Bendelac, D. R. Littman, Genetic evidence supporting selection of the Valpha14i NKT cell lineage from double-positive thymocyte precursors. *Immunity* **22**, 705–716 (2005).
18. Z. Sun, D. Unutmaz, Y. R. Zou, M. J. Sunshine, A. Pierani, S. Brenner-Morton, R. E. Mebius, D. R. Littman, Requirement for ROR γ in thymocyte survival and lymphoid organ development. *Science* **288**, 2369–2373 (2000).
19. R. Wang, H. Xie, Z. Huang, J. Ma, X. Fang, Y. Ding, Z. Sun, Transcription factor network regulating CD⁺CD8⁺ thymocyte survival. *Crit. Rev. Immunol.* **31**, 447–458 (2011).
20. H. Xie, M. S. Sadim, Z. Sun, ROR γ t recruits steroid receptor coactivators to ensure thymocyte survival. *J. Immunol.* **175**, 3800–3809 (2005).
21. D. Komander, M. Rape, The ubiquitin code. *Annu. Rev. Biochem.* **81**, 203–229 (2012).
22. S. Rutz, N. Kayagaki, Q. T. Phung, C. Eidsenchen, R. Noubade, X. Wang, J. Lesch, R. Lu, K. Newton, O. W. Huang, A. G. Cochran, M. Vasser, B. P. Fauber, J. DeVoss, J. Webster, L. Diehl, Z. Modrusan, D. S. Kirkpatrick, J. R. Lill, W. Ouyang, V. M. Dixit, Deubiquitinase DUBA is a post-translational brake on interleukin-17 production in T cells. *Nature* **518**, 417–421 (2015).
23. L. Han, J. Yang, X. Wang, Q. Wu, S. Yin, Z. Li, J. Zhang, Y. Xing, Z. Chen, A. Tsun, D. Li, M. Piccioni, Y. Zhang, Q. Guo, L. Jiang, L. Bao, L. Lv, B. Li, The E3 deubiquitinase USP17 is a positive regulator of retinoic acid-related orphan nuclear receptor γ t (ROR γ t) in Th17 cells. *J. Biol. Chem.* **289**, 25546–25555 (2014).
24. X. Wang, J. Yang, L. Han, K. Zhao, Q. Wu, L. Bao, Z. Li, L. Lv, B. Li, TRAF5-mediated Lys-63-linked polyubiquitination plays an essential role in positive regulation of ROR γ t in promoting IL-17A expression. *J. Biol. Chem.* **290**, 29086–29094 (2015).
25. J. Yang, P. Xu, L. Han, Z. Guo, X. Wang, Z. Chen, J. Nie, S. Yin, M. Piccioni, A. Tsun, L. Lv, S. Ge, B. Li, Cutting edge: Ubiquitin-specific protease 4 promotes Th17 cell function under inflammation by deubiquitinating and stabilizing ROR γ t. *J. Immunol.* **194**, 4094–4097 (2015).
26. H. Hu, S. C. Sun, Ubiquitin signaling in immune responses. *Cell Res.* **26**, 457–483 (2016).
27. R. Holmes, J. C. Zúñiga-Pflücker, The OP9-DL1 system: Generation of T-lymphocytes from embryonic or hematopoietic stem cells in vitro. *Cold Spring Harb. Protoc.* **2009**, pdb.prot5156 (2009).
28. E. Bettelli, M. Pagany, H. L. Weiner, C. Lington, R. A. Sobel, V. K. Kuchroo, Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *J. Exp. Med.* **197**, 1073–1081 (2003).
29. L. Wu, K. E. R. Hollinshead, Y. Hao, C. Au, L. Kroehling, C. Ng, W. Y. Lin, D. Li, H. M. Silva, J. Shin, J. J. Lafaille, R. Possemato, M. E. Pacold, T. Papagiannakopoulos, A. C. Kimmelman, R. Satija, D. R. Littman, Niche-selective inhibition of pathogenic Th17 cells by targeting metabolic redundancy. *Cell* **182**, 641–654.e20 (2020).
30. I. M. Stromnes, J. M. Goverman, Active induction of experimental allergic encephalomyelitis. *Nat. Protoc.* **1**, 1810–1819 (2006).
31. Z. He, J. Zhang, Q. Du, J. Xu, Y. Gwack, Z. Sun, SRC3 is a cofactor for ROR γ t in Th17 differentiation but not thymocyte development. *J. Immunol.* **202**, 760–769 (2019).
32. R. Wang, H. Xie, Z. Huang, J. Ma, X. Fang, Y. Ding, Z. Sun, T cell factor 1 regulates thymocyte survival via a ROR γ t-dependent pathway. *J. Immunol.* **187**, 5964–5973 (2011).

33. D. J. Cua, J. Sherlock, Y. Chen, C. A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S. A. Lira, D. Gorman, R. A. Kastelein, J. D. Sedgwick, Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* **421**, 744–748 (2003).
34. K. Xu, N. Yin, M. Peng, E. G. Stamatziades, S. Chhangawala, A. Shyu, P. Li, X. Zhang, M. H. Do, K. J. Capistrano, C. Chou, C. S. Leslie, M. O. Li, Glycolytic ATP fuels phosphoinositide 3-kinase signaling to support effector T helper 17 cell responses. *Immunity* **54**, 976–987.e7 (2021).
35. J. T. Gaublotte, N. Yosef, Y. Lee, R. S. Gertner, L. V. Yang, C. Wu, P. P. Pandolfi, T. Mak, R. Satija, A. K. Shalek, V. K. Kuchroo, H. Park, A. Regev, Single-cell genomics unveils critical regulators of Th17 cell pathogenicity. *Cell* **163**, 1400–1412 (2015).
36. M. Ciofani, A. Madar, C. Galan, M. Sellars, K. Mace, F. Pauli, A. Agarwal, W. Huang, C. N. Parkurst, M. Muratet, K. M. Newberry, S. Meadows, A. Greenfield, Y. Yang, P. Jain, F. K. Kirigin, C. Birchmeier, E. F. Wagner, K. M. Murphy, R. M. Myers, R. Bonneau, D. R. Littman, A validated regulatory network for Th17 cell specification. *Cell* **151**, 289–303 (2012).
37. Y. Wang, J. Godec, K. Ben-Aissa, K. Cui, K. Zhao, A. B. Pucsek, Y. K. Lee, C. T. Weaver, R. Yagi, V. Lazarevic, The transcription factors T-bet and Runx are required for the ontogeny of pathogenic interferon- γ -producing T helper 17 cells. *Immunity* **40**, 355–366 (2014).
38. P. R. Mangan, L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, C. T. Weaver, Transforming growth factor- β induces development of the Th17 lineage. *Nature* **441**, 231–234 (2006).
39. M. Veldhoen, R. J. Hocking, C. J. Atkins, R. M. Locksley, B. Stockinger, TGF β in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* **24**, 179–189 (2006).
40. P. Miossec, T. Korn, V. K. Kuchroo, Interleukin-17 and type 17 helper T cells. *N. Engl. J. Med.* **361**, 888–898 (2009).
41. T. Korn, E. Bettelli, M. Oukka, V. K. Kuchroo, IL-17 and Th17 Cells. *Annu. Rev. Immunol.* **27**, 485–517 (2009).
42. T. Okuda, M. Nishimura, M. Nakao, Y. Fujita, RUNX1/AML1: A central player in hematopoiesis. *Int. J. Hematol.* **74**, 252–257 (2001).
43. T. Egawa, R. E. Tillman, Y. Naoe, I. Taniuchi, D. R. Littman, The role of the Runx transcription factors in thymocyte differentiation and in homeostasis of naive T cells. *J. Exp. Med.* **204**, 1945–1957 (2007).
44. T. Okuda, J. van Deursen, S. W. Hiebert, G. Grosveld, J. R. Downing, AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* **84**, 321–330 (1996).
45. R. Sood, Y. Kamikubo, P. Liu, Role of RUNX1 in hematological malignancies. *Blood* **129**, 2070–2082 (2017).
46. V. Lazarevic, X. Chen, J. H. Shim, E. S. Hwang, E. Jang, A. N. Bolm, M. Oukka, V. K. Kuchroo, L. H. Glimcher, T-bet represses Th17 differentiation by preventing Runx1-mediated activation of the gene encoding ROR γ t. *Nat. Immunol.* **12**, 96–104 (2011).
47. L. Li, N. Patsoukis, V. Petkova, V. A. Boussiotis, Runx1 and Runx3 are involved in the generation and function of highly suppressive IL-17-producing T regulatory cells. *PLOS ONE* **7**, e45115 (2012).
48. M. Martinez, M. Hinojosa, D. Trombly, V. Morin, J. Stein, G. Stein, A. Javed, S. E. Gutierrez, Transcriptional auto-regulation of RUNX1 P1 promoter. *PLOS ONE* **11**, e0149119 (2016).
49. K. Yasuda, Y. Takeuchi, K. Hirota, The pathogenicity of Th17 cells in autoimmune diseases. *Semin. Immunopathol.* **41**, 283–297 (2019).
50. K. Ghoreschi, A. Laurence, X. P. Yang, C. M. Tato, M. J. McGeachy, J. E. Konkel, H. L. Ramos, L. Wei, T. S. Davidson, N. Bouladoux, J. R. Grainger, Q. Chen, Y. Kanno, W. T. Watford, H. W. Sun, G. Eberl, E. M. Shevach, Y. Belkaid, D. J. Cua, W. Chen, J. J. O'Shea, Generation of pathogenic Th17 cells in the absence of TGF- β signalling. *Nature* **467**, 967–971 (2010).
51. C. L. Langrish, Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, D. J. Cua, IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* **201**, 233–240 (2005).
52. G. Tonel, C. Conrad, U. Laggner, P. DiMeglio, K. Grys, T. K. McClanahan, W. M. Blumenschein, J. Z. Qin, H. Xin, E. Oldham, R. Kastelein, B. J. Nickoloff, F. O. Nestle, Cutting edge: A critical functional role for IL-23 in psoriasis. *J. Immunol.* **185**, 5688–5691 (2010).
53. C. Fotiadou, E. Lazaridou, E. Sotiriou, D. Ioannides, Targeting IL-23 in psoriasis: Current perspectives. *Psoriasis (Auckl)* **8**, 1–5 (2018).
54. J. Hanzel, G. R. D'Haens, Anti-interleukin-23 agents for the treatment of ulcerative colitis. *Expert Opin. Biol. Ther.* **20**, 399–406 (2020).
55. A. R. Moschen, H. Tilg, T. Raine, IL-12, IL-23, and IL-17 in IBD: Immunobiology and therapeutic targeting. *Nat. Rev. Gastroenterol. Hepatol.* **16**, 185–196 (2019).
56. B. M. Segal, C. S. Constantinescu, A. Raychaudhuri, L. Kim, R. Fidelus-Gort, L. H. Kasper, Ustekinumab MS Investigators, Repeated subcutaneous injections of IL12/23 p40 neutralising antibody, ustekinumab, in patients with relapsing-remitting multiple sclerosis: A phase II, double-blind, placebo-controlled, randomised, dose-ranging study. *Lancet Neurol.* **7**, 796–804 (2008).
57. C. Sheridan, Footrace to clinic heats up for T-cell nuclear receptor inhibitors. *Nat. Biotechnol.* **31**, 370 (2013).
58. E. Ueda, S. Kurebayashi, M. Sakaue, M. Backlund, B. Koller, A. M. Jetten, High incidence of T-cell lymphomas in mice deficient in the retinoid-related orphan receptor ROR γ . *Cancer Res.* **62**, 901–909 (2002).
59. M. Liljevald, M. Rehnberg, M. Soderberg, M. Ramnegard, J. Borjesson, D. Luciani, N. Krutro, L. Branden, C. Johansson, X. Xu, M. Bjursell, A. K. Sjogren, J. Hornberg, U. Andersson, D. Keeling, J. Jirholt, Retinoid-related orphan receptor γ (ROR γ) adult induced knockout mice develop lymphoblastic lymphoma. *Autoimmun. Rev.* **15**, 1062–1070 (2016).

Acknowledgments: We thank J. C. Zuniga-Pflucker (University of Toronto) for the DP9-DL4 stroma cell line, W.S. Pear (University of Pennsylvania) for the retroviral vector MIGR1, I. Taniuchi for the MSCV-Runx1 retroviral vector, Y.C. Yuan (Bioinformatics Core of City of Hope) for the guidance of Partek Flow, and Biocytogen for assisting with the design and generation of ROR γ ^{K256R/K256R} mice. We also thank the following City of Hope core services: Animal Resource Center, Integrative Genomics Core, Flow Cytometer Core, Mass Spectrometry Core, and Bioinformatics Core. We thank C. S. Jayasena for reviewing and editing the manuscript. **Funding:** This work was supported by grants from NIH R01-AI109644, R21-AI163256, institutional pilot funding, Jackie and Bruce Barrow Cancer Research Scholars' Program, and Caltech-CoH Biomedical Initiative. The research reported in this publication included work performed in the animal, genomic, flow cytometry, and mass spectrometry cores supported under NIH grant P30CA033572. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. **Author contributions:** Conceptualization: Z.S., Z.H., and X.Z. Methodology: X.Z., H.W., W.Z., Z.H., Y.G., N.J., and K.O.L. Investigation: X.Z., H.W., W.Z., Z.H., and K.O.L. Visualization: X.Z., H.W., W.Z., Z.H., and K.O.L. Funding acquisition: Z.S. Supervision: Z.S. Writing—original draft: Z.S., X.Z., and H.W. Writing—review and editing: Z.S., X.Z., H.W., Y.G., N.J., and Z.H. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. RNA-seq and ChIP-seq data is available on Gene Expression Omnibus (GEO) repository (accession #: GSE211414 and GSE211509).

Submitted 9 May 2022

Accepted 7 September 2022

Published 21 October 2022

10.1126/sciadv.adc9221