

Supporting Information

SI Materials and Methods

Mice

Rosa-M2rtTA, TRE-REV-ERB α , and 2D2 transgenic mice were purchased from Jackson Laboratory. The three transgenic lines were crossed to generate Rosa-M2rtTAxTRE-RVBx2D2 triple transgenic mice. REV-ERB $\alpha^{fl/fl}/\beta^{fl/fl}$ mice were generated previously (1). CD4Cre transgenic, C57BL/6, SJL/J, and Ly5.1⁺ congenic mice were purchased from the Jackson Laboratory. All mice were maintained in the Salk Institute SPF animal facility in accordance with institutional regulations.

In vitro CD4 T cell differentiation

For mouse CD4 T cell differentiation, total CD4 T cells were isolated from the spleen and lymph nodes using the Dynabeads CD4 Positive Isolation Kit (Invitrogen). When indicated, enriched CD4 T cells were further sorted for naive cells (CD25- CD62L low CD44 high). Cells were resuspended in Click's medium (Irvine Scientific) at 1 million cells per ml, and then plated in 24 well plates coated with Goat-Hamster IgG antibody (200ng/ml; MP Biomedicals) with the addition of soluble anti-CD3 (1 μ g/ml; 145-2C11) and anti-CD28 (1 μ g/ml; 37.51) from Bio X Cell. Polarizing conditions for different T helper subsets are as following: Th1: mIL-2 (100U/ml; Tonbo), mIL-12 (20ng/ml; Peprotech) and anti-IL-4 (5 μ g/ml; Bio X Cell). Th2: mIL-2 (100U/ml; Tonbo), mIL-4 (20ng/ml; Biolegend), anti-IFN- γ and anti-IL-12 (5 μ g/ml; Bio X Cell). Th17: mIL-6 (20ng/ml; Biolegend), mIL-23 (20ng/ml; R&D), mIL-1 β (20ng/ml; Peprotech), hTGF- β

(2ng/ml; Peprotech), anti-IFN- γ and anti-IL-12 (5 μ g/ml; Bio X Cell). iTreg: mIL-2 (100U/ml; Tonbo) and hTGF- β (2ng/ml; Peprotech).

Primary human CD4 T cells for all experiments were sourced from de-identified blood donors (TSRI Normal Blood Donor Service, La Jolla, CA) following written informed consent in accordance with the Declaration of Helsinki. Study approval was obtained from the TSRI institutional review board (IRB). For human CD4 T cell differentiation, total CD4 T cells were isolated from PBMC using the EasySep Human CD4⁺ T Cell Enrichment Kit (StemCell). Cells were plated at 1 million per ml in 24 well plates coated with 5ug/ml anti-human CD3 and anti-human CD28 (Tonbo). For human Th1 differentiation, hIL-2 (100U/ml; Tonbo), hIL-12 (20ng/ml; Tonbo), anti-human IL-4 (10ug/ml; Biolegend) were added to culture. For human Th17 differentiation, hIL-6 (20ng/ml; Tonbo), hIL-23 (20ng/ml; Tonbo), hIL-1 β (20ng/ml; Tonbo), hTGF- β (5ng/ml; Peprotech), anti-human IL-4 and anti-human IFN- γ (10 μ g/ml; Biolegend) were added to culture. Cells were cultured for 6-8 days and then analyzed by FACS and RT-qPCR.

Flow cytometry

Cytokine expression was assessed by intracellular staining. The following antibodies were used for cell surface staining: anti-CD4-PerCPCy5.5 (RM4-5) and anti-CD62L-APC(MEL-14) from Tonbo Biosciences; anti-CD44PE(IM7) and anti-CD25-FITC(7D4) from Ebiosciences; for intracellular staining: anti-IL-17A-PE(eBio17B7), anti-IFN- γ -APC(XMG1.2) and anti-Foxp3-FITC(FJK-16s) from Ebiosciences. Cells were stimulated with

PMA and ionomycin in the presence of brefeldin A (GolgiPlug; BD) for 5 hours at 37°C. Cells were then fixed with fixation and permeabilization reagents from BD or Ebioscience (for Foxp3 staining) and labeled with appropriate antibodies before being analyzed on a BD FACS Aria Cell Sorter. Results were analyzed with FlowJo (Tree Star).

Reverse transcription and quantitative PCR

Total RNA was isolated from CD4 T cells using TRIzol reagent (Life technologies). cDNA was synthesized with iScript Reverse Transcription Supermix for RT-qPCR (Bio-rad) according to the manufacturer's protocol, followed by qPCR using SYBR Green PCR Master Mix (Applied Biosystems). Quantitative PCR was performed on an Applied Biosystems ViiA™ 7 Real-Time PCR System with gene specific primers listed in Table S1. The comparative threshold cycle method and an internal control (β -actin) were used to normalize the expression of genes of interest.

Western blot

Proteins from cell lysates were electrophoresed on a 10% SDS PAGE gel and transferred to PVDF membrane. Blots were probed with anti-T-bet (1:1,000; BioLegend), anti-ROR γ t (1:500; Santa Cruz), anti-REV-ERB α (1:1000; described previously(1)), or anti-REV-ERB β (1:100; described previously(1)) antibodies, at 4 °C overnight followed by incubation for 1 h at room temperature with the appropriate secondary antibodies conjugated to horseradish peroxidase. Expression of β -Actin was used as loading control. Detection was performed using the SuperSignal West Femto

Maximum sensitivity Substrate kit (Thermo scientific) following the manufacturer's protocol.

Retroviral transduction

HEK 293T cells were seeded at 0.5 million per well of a six well plate the day before transfection. 2 μ g total plasmid DNA was transfected via Fugene6 reagent (Promega) according to manufacturer's protocol, which contained 0.8 μ g of pCL-Eco retroviral packaging plasmid and 1.2 μ g of expression plasmid. pCL-Eco was a gift from Inder Verma(2). Viral supernatant was harvested 48 and 72 hours post transfection. CD4+ T cells were cultured in Th17 polarizing condition and retroviral transduction was performed 24 and 48 hours post activation by incubating cells with viral supernatant in the presence of polybrene (4 μ g/ml; Millipore) and centrifuged at 2500 rpm for 90 minutes at 32°C.

Dual luciferase reporter assay

Mouse EL4 T cells were transfected using the Neon transfection system (Thermo Fisher Scientific) according to manufacturer's protocol. Luciferase assays were performed at 48 hours post transfection using the Dual-Glo Lucifer's Assay System (Promega) according to the manufacturer's protocol. The luciferase reporter pGL4 mL-17 2kb promoter+CNS-5 was purchased from Addgene (plasmid #20128)(3).

DNA binding assay

Primary mouse CD4 T cells were transduced twice with retroviral vectors carrying Flag-tagged REV-ERB α , or HA-tagged ROR γ t as previously described. 48 hours after second transduction, cells were harvested for nuclear protein extraction. The nuclear proteins were incubated with streptavidin agarose (Millipore) and biotinylated RORE oligonucleotide for 2 hours at 4°C, followed by washes. Proteins bound to agarose beads were assayed by Western blot with anti-Flag antibody (M2; Sigma) and anti-HA antibody (HA-7; Sigma). The sequence of biotinylated Il17a CNS5 RORE oligonucleotide is 5'-TCAGTTGCTGACCTTGATTCTA-3'.

Chromatin Immunoprecipitation

Naive CD4⁺ T cells were activated and polarized in Th17 condition for 3 days for ChIP experiments as described previously(1). All antibodies were used at 4 μ g/reaction.

Mouse IgG control antibody was purchased from Santa Cruz Biotechnology. ROR γ t ChIP was performed with a combination of antibodies from Biolegend and Santa Cruz Biotechnologies. REV-ERB α antibody was generated as previously described(1).

NCoR1 antibody was purchased from Cell Signaling. Primers spanning the regulatory regions of *Il17a*, *Cry1*, and *Gmpr* are described in Table S2. Ct value for each sample was normalized to the corresponding input value.

ChIP-sequencing and data analysis

ChIP-sequencing libraries were constructed and sequenced as described previously(1).

Short DNA reads were demultiplexed using Illumina CASAVA v1.8.2. Reads were aligned against the mouse mm9 reference genome using the Bowtie2 aligner with

standard parameters that allow up to 2 mismatches in the read. Peak calling, motif analyses, and other data analysis were performed using HOMER, a software suite for ChIP-seq analysis as described previously(1). Visualization of ChIP-Seq results was achieved by uploading custom tracks onto the UCSC genome browser. ChIP-seq data can be accessed in the NCBI GEO database under the accession GSE72271.

RNA-sequencing and data analysis

mRNA was extracted from Th17 cells on day 3 of *in vitro* differentiation. RNA-sequencing libraries were prepared from 100ng total RNA (TrueSeq v2, Illumina) and single-ended sequencing performed on the Illumina HiSeq 2500, using bar-coded multiplexing and a 50 bp read length, yielding a median of 34.1M reads per sample. Read alignment and junction finding was accomplished using STAR(4) and differential gene expression with Cuffdiff 2(5), utilizing UCSC mm9 as the reference sequence. Student's t test was performed to generate a list of differentially expressed genes ($p < 0.05$), which was then run through KEGG pathway analysis on DAVID(6, 7) to examine enriched functional groups. Heatmaps were generated on Matrix2png(8). RNA-seq data can be accessed in the NCBI Sequence Read Archive under the accession #SRP062715.

EAE models

For active EAE, mice were immunized subcutaneously with 200ng of MOG(35-55) peptide (BL6 mice) or PLP(139-151) peptide (SJL mice) in CFA, and received 200ng of Pertussis toxin intra-peritoneally on days 0 and 2. Mice were monitored daily for disease

progression using the following scoring criteria: 0, normal; 1, limp tail; 2, weakened hind limbs; 3, complete paralysis of one limb; 4- complete paralysis of both limbs with weakening of the front limbs. At the end point, half of the brain and spinal cord were preserved for histology. Lymphocytes were enriched from the other half of the tissues by Percoll gradient separation and analyzed for cytokine production by flow cytometry after 5 hours of PMA stimulation in the presence of Ionomycin and Golgi-plug.

For passive EAE, CD4 T cells from Rosa-M2rtTAxTRE-RVBx2D2 mice were activated with plate-bound Goat-hamster IgG and soluble anti-CD3 and anti-CD28 under Th17 condition. After 3 days of culturing, the cells were re-stimulated overnight in the presence of IL-18 (20ng/ml; Fisher Scientific). In the following day, 2-3 million cells were adoptively transferred into wild type recipient mice. The recipient mice were given normal water or Doxycycline water to induce REV-ERB α expression in transferred T cells. EAE disease progression was monitored as in active EAE model. At the end point, mice were harvested for histology and T cell FACS profiling. Transferred 2D2 T cells and host T cells were distinguished by the expression of TCR Va3.2.

Histology analysis

Mice were euthanized by CO₂ asphyxia and perfused with PBS. The brains and spines were dissected and post-fixed in 10% buffered formalin for at least 72 hours before processing. Vertebral columns with spinal cords were decalcified before sectioning *in situ*. Multiple transverse sections of the cervical, thoracic, and lumbar spinal cord were

prepared routinely and stained with H&E and Luxol Fast Blue. Histological sections were evaluated for inflammation and neuronal degeneration.

SR9009 treatment

For cell cultures, SR9009 was dissolved in DMSO and added at final concentrations of 5 μ M. For EAE experiments, SR9009 was dissolved in 15% Cremaphor and 85% water at 10mg/ml. Mice were administered at 100 mg/kg body weight intraperitoneally twice daily.

References for SI Materials and Methods

1. Cho H, et al. (2012) Regulation of circadian behaviour and metabolism by REV-ERB- α and REV-ERB- β . *Nature* 485(7396):123–127.
2. Naviaux RK, Costanzi E, Haas M, Verma IM (1996) The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *J Virol* 70(8):5701–5705.
3. Zhang F, Meng G, Strober W (2008) Interactions among the transcription factors Runx1, RORgammat and Foxp3 regulate the differentiation of interleukin 17-producing T cells. *Nat Immunol* 9(11):1297–1306.
4. Dobin A, et al. (2013) STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29(1):15–21.
5. Trapnell C, et al. (2013) Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat Biotechnol* 31(1):46–53.
6. Huang DW, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4(1):44–57.
7. Huang DW, Sherman BT, Lempicki RA (2009) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 37(1):1–13.
8. Pavlidis P, Noble WS (2003) Matrix2png: a utility for visualizing matrix data. *Bioinformatics* 19(2):295–296.

Supplementary Figure Legends

Figure S1. REV-ERBs inhibit ROR γ t-dependent IL-17A expression. CD4⁺ T cells were cultured in the presence of IL-2, α -IL-12 and α -IFN- γ , and transduced with a MIGR1 control vector, ROR γ t, or ROR γ t with either REV-ERB α or REV-ERB β at a 1:1 ratio. Cytokine production was examined on day 4 by flow cytometry. FACS plots shown are representative of 3 independent experiments. Data represents mean \pm s.e.m. Statistical analyses were performed using unpaired two-tailed Student's t test (*p<0.05).

Figure S2. REV-ERB α and ROR γ t both bind to Th17 signature genes in ChIP-seq analysis. Trace analysis of ChIP-seq data visualized on the UCSC genome browser showing overlapping binding sites of REV-ERB α and ROR γ t at *Il23r*, *Tgfb3* and *Cry1* loci.

Figure S3. Doxycycline treatment induces transgenic REV-ERB α expression in T cells *in vivo*, and does not affect T cell homing property. (a) Relative expression of transgenic REV- ERB α in adoptively transferred triple transgenic CD4⁺ T cells sorted from the brain and spinal cord of mice treated with or without doxycycline. (b) CD4⁺ T cell percentage of host and transferred triple transgenic 2D2 cells in the spleen and CNS at EAE disease endpoint. (c) EAE disease clinical scores of mice that received adoptive transfer of *in vitro* differentiated wild-type 2D2 Th17 cells, then treated with or without doxycycline (n=8 per group). Data represents mean \pm s.e.m. Statistical analyses were performed using unpaired two-tailed Student's t test (*p<0.05).

Figure S4. SR9009 treatment does not skew thymic T cell development or affect the activation state of periphery T cells. Wildtype mice were injected with SR9009

(100mg/kg body weight, b.i.d.) or vehicle control (n=3 per group) for 5 days before analyzed for T cell population in the thymus (a) and spleen (b) and (c). Data represents mean \pm s.e.m. Statistical analyses were performed using unpaired two-tailed Student's t test (n.s. not significant, $p>0.05$).

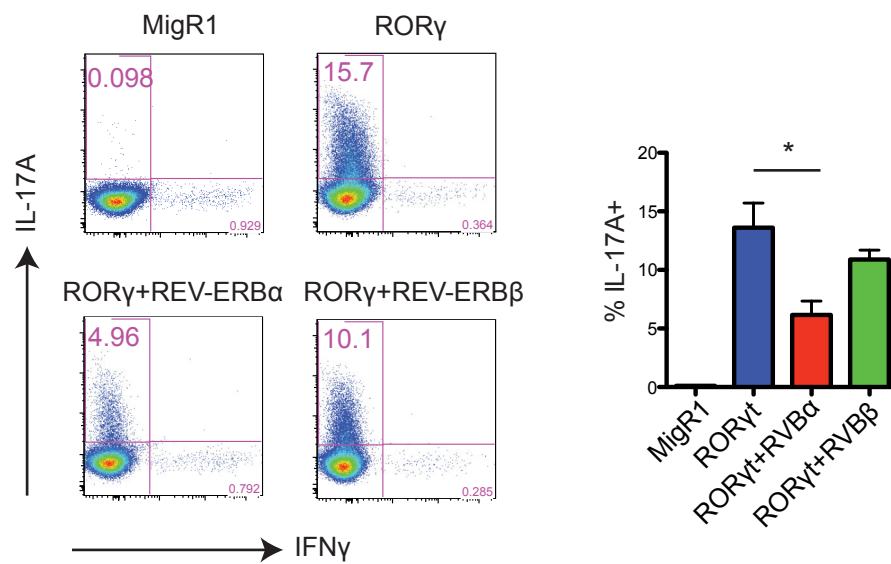


Figure S1

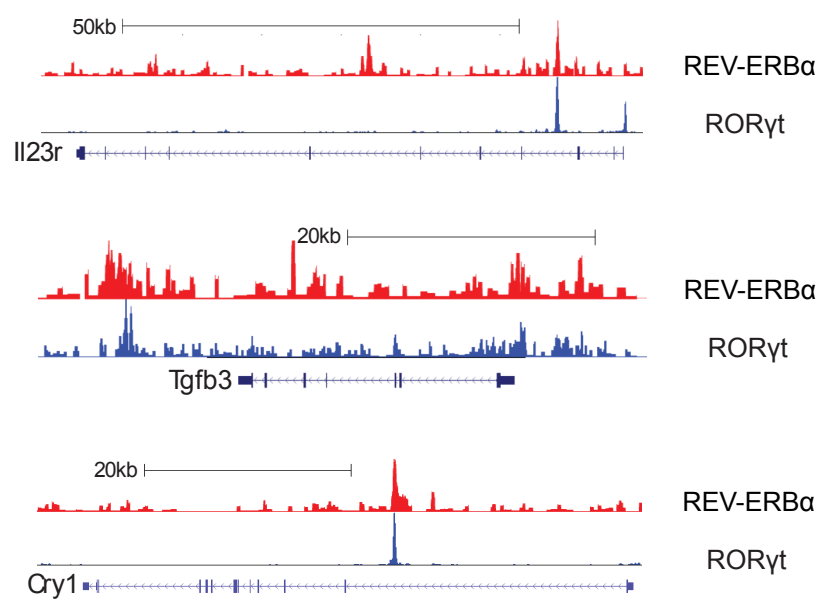


Figure S2

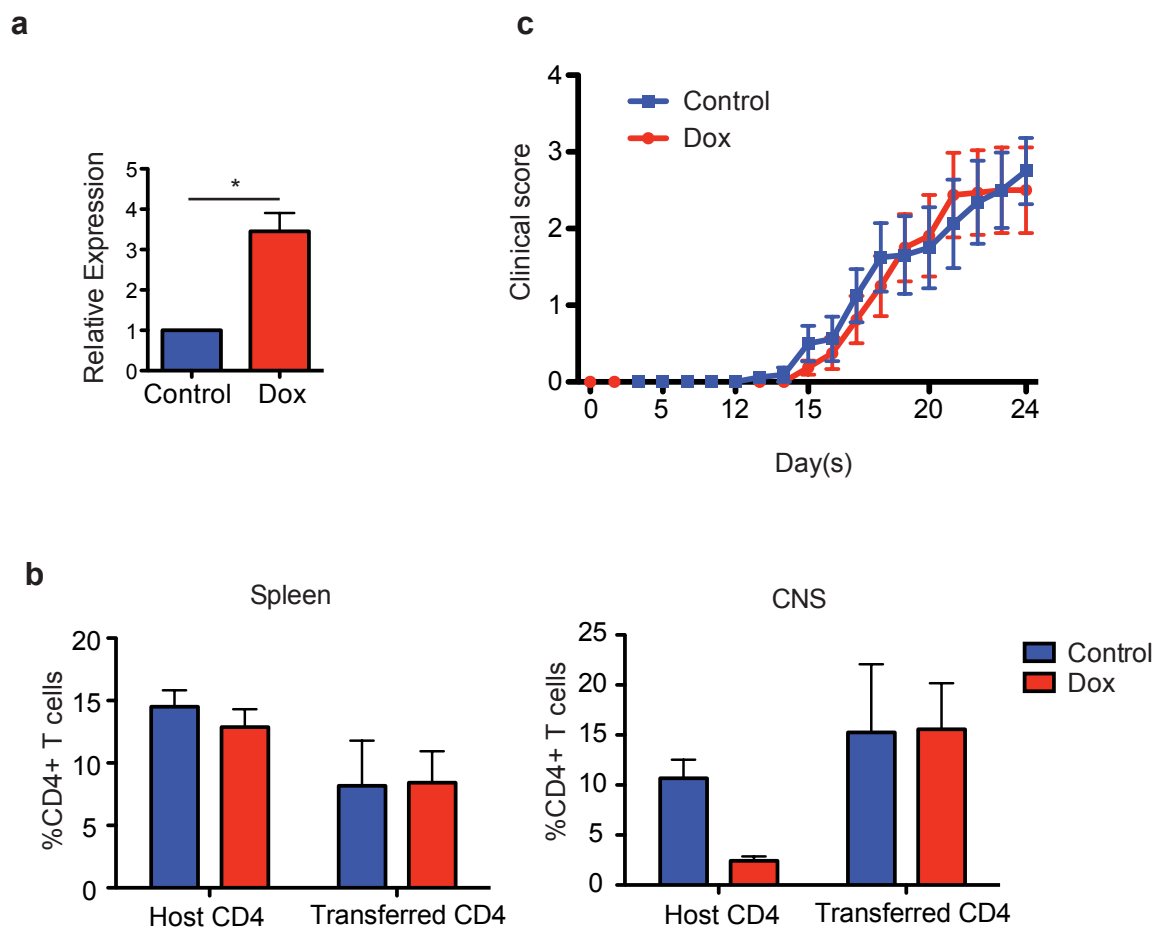


Figure S3

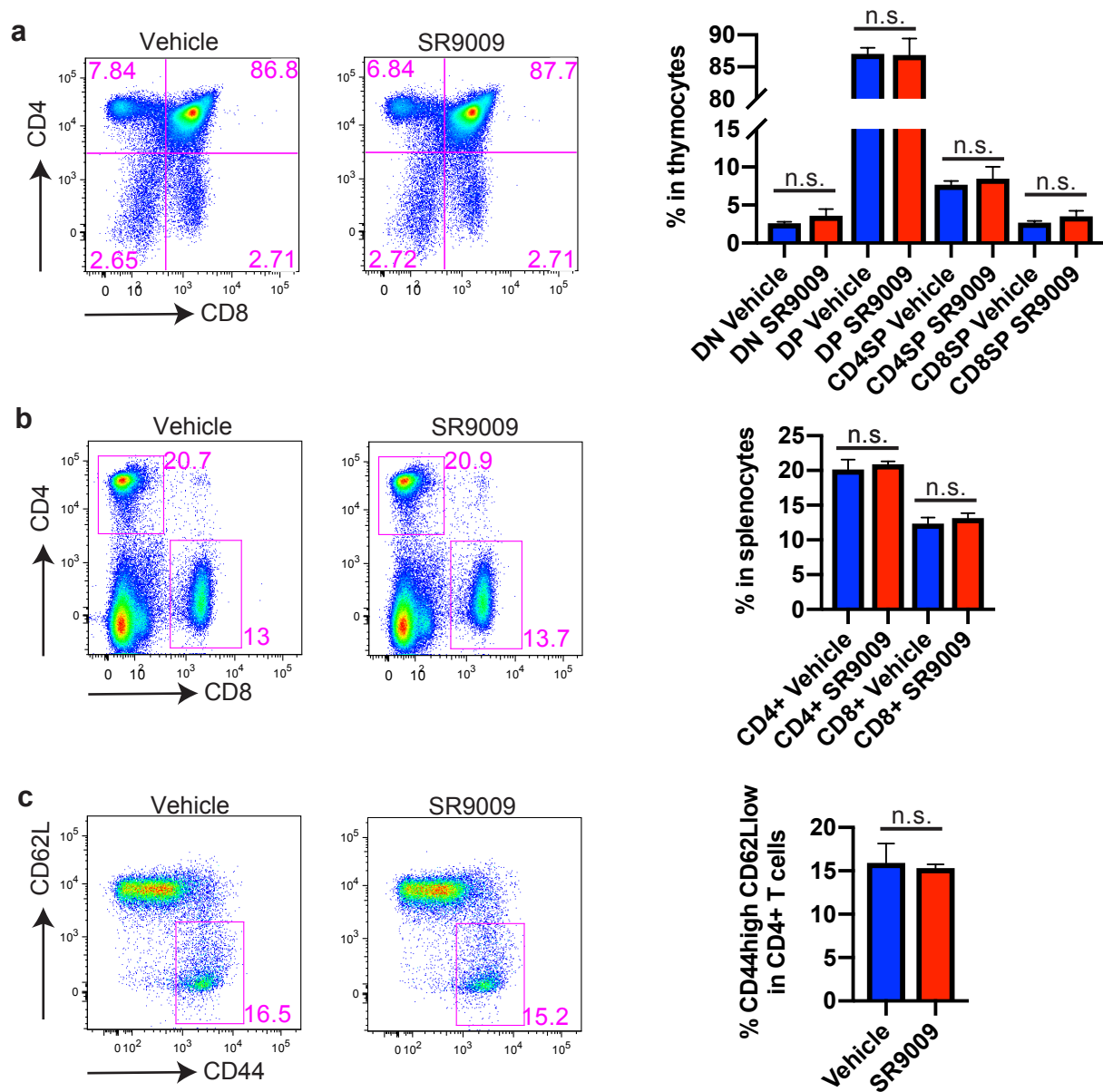


Figure S4

Table S1. Sequences of RT-qPCR primers.

Gene	Forward (5'-3')	Reverse (5'-3')
Actin	CTGTCGAGTCGCGTCCACCCG	CACATGCCGGAGCCGTTGTCGAC
Rorgt	CCGCTGAGAGGGCTTCAC	TGCAGGAGTAGGCCACATTACA
Gata3	CTTCCCACCCAGCAGCCTGC	CGGTACCATCTCGCCGCCAC
Tbx21	GTCGCGCTCAGCAACCACCT	CGGCCACGGTGAAGGACAGG
Nr1d1	GGGCACAAGCAACATTACCA	CACGTCCCCACACACCTTAC
Nr1d2	TGGGACTTTTGAGGTTTTAATGG	GTGACAGTCCGTTTCCTTTGC
Foxp3	GCGAAGGGCTCGGTAGTCCTCA	TTCAGTAGAGGGGCAGCCTGCAG
Ifng	ACAGCCAAGCGGCTGACTGAAC	CTGTTAAAGCGCTGGCCCGGA
Il17a	CCAGCTGATCAGGACGCGCA	TCACTGTAGCCGCCAGGCTCA
Il17f	GAGGATAAACTGTGAGAGTTGAC	GAGTTCATGGTGCTGTCTTCC
Il23r	GCCAAGAGAACCATTCCCGA	TCAGTGCTACAATCTTCAGAGGAA
Nfil3	CTTTCAGGACTACCAGACATCCAA	GATGCAACTTCCGGCTACCA
Rat Nr1d1	CATGGTGCTACTGTGTAAAGTGTGT	CGCAGGCATGCACGCCATAG
Human Nr1d1	TTAAGGCTGGCACCTTTGAG	GAAGTCGAACATGGCACTGA
Human Nr1d2	TGGCGTCAGGATTCCACTAT	AGCGACATTGCTGACATCTG
Human Rorgt	GCACTTTTCCGAGGATGAGA	ATGCTTTGGCGATGAGTCTT
Human Tbx21	GGATGCGCCAGGAAGTTTCA	GGAGCACAATCATCTGGGTCA
Human Actin	CAGTCACCATAACCGGGAAAA	TTGCTGTCCTCCCTAGAACTC

Table S2. Sequences of ChIP-qPCR primers.

Gene	Forward (5'-3')	Reverse (5'-3')
Il17a	CCGTTTAGACTTGAAACCCAGTC	GCAGCAGCTTCAGATATGTCC
Cry1	GTCTCTAGGGTCGACACTGTATGG TT	AGTGGGTGGTTTAGCATACTCCC ATC
Gmpr	CAGCTGGAACAGCCTTGGA	AAATGTCAAGGCCCTGTGA