Supplementary Information

Methyltransferase Setd2 prevents T cell-mediated autoimmune diseases via phospholipid remodeling

Yali Chen[a,b], Kun Chen[a,c], Ha Zhu[b], Hua Qin[d], Juan Liu[b, 1] and Xuetao Cao[a, b, d, 1]

*Corresponding author. Email: juanliu@immunol.org (J.L.), caoxt@immunol.org (X. C.)

This PDF file includes:

Supplementary information text

Materials and Methods

Reagents

Antibodies used for flow cytometry, including anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-CD3 (17A2), anti-CD45 (30-F11), anti-CD25 (PC61), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-B220 (RA3-6B2), anti-IL-17A (TC11-18H10), anti-Foxp3 (150D/E4), anti-IL4 (11B11) were obtained from Biolegend. anti-IFNγ (XMG1.2) was from eBioscience. For mouse T cell stimulation, anti-CD3 (145-2C11) and anti-CD28 (37.51) were from BD; recombinant mouse IL-2 (402-ML), IL-6 (406-ML), IL-1β (401-ML), IL-23 (1887-ML), IL-4 (404-ML), IL-12 (419-ML) and recombinant human TGF-β1 (7754-BH) were from R&D Systems; neutralizing anti-mouse IFN-γ (XMG1.2) was from Biolegend and anti-mouse IL-4 (11B11) was from BioXcell. Inhibitors used in this study, 4-phenylbutyric acid (4-PBA), CI-976, and N-acetyl cysteine (NAC) were obtained from MedChemExpress.

T cell differentiation

$CD4^+$ T cells were purified from the spleen or mLN using a $CD4^+$ T Cell Isolation Kit (Miltenyi), with the purity of $CD4^+$ T cells > 95%. The culture conditions for Th17 and iTreg cells were as follows and as described previously (1). Th17 cell differentiation conditions: 30 ng/ml IL-6, 3 ng/ml h-TGF-β1, 10 ng/ml IL-1β, 20 ng/ml IL-23, 10 μg/ml anti-IFNγ and 10 μg/ml anti-IL4. iTreg cell differentiation conditions: 10 ng/ml IL-2, 10 ng/ml h-TGF-β1, 10 μg/ml anti-IFNγ and 10 μg/ml anti-IL4. Th1 cell differentiation conditions: 20 ng/ml IL-12 and 10 μg/ml anti-IL4. Th2

cell differentiation conditions: 20 ng/ml IL-4 and 10 μg/ml anti-IFNγ. After three days of stimulation, cells were collected for later analysis.

Cell proliferation assay

$CD4^+$ T cells were labeled with Cell Trace Violet (Thermo Fisher Scientific) at 2.5μM for 10 min at 37 °C and then stimulated with anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml) for three days. Cell proliferation was detected using the Fortessa flow cytometer (BD Bioscience).

Flow cytometry

For surface staining of T cells (CD3, CD4, CD8, CD25, CD44, and CD62L), and B cells (B220), the cells were stained with the antibody in PBS for 20 min at room temperature. For intracellular staining, T cells were fixed with 4% paraformaldehyde, and stained with Foxp3 or IL-17 antibody in 1× Perm (BD Bioscience). Cells were then detected with Fortessa flow cytometer (BD Bioscience) and analyzed using FACS Diva software (BD Bioscience) or FlowJo software (Tree Star).

CBA analysis

The concentration of IL-17A, IL-17F, IFN-γ, and IL-10 concentrations in T cells was measured by CBA following the manufacturer's instructions (BD Bioscience).

Cell Rox/ER Tracker staining

Th17 or iTreg cells were incubated with 1μM CellROX Deep Red Reagent (Thermo Scientific) for 40 min or with 1 μM ER Tracker (Molecular Probes) for 20 min at 37 °C and then stained with CD4, IL-17A and Foxp3 antibodies at 4 °C, protected from light.

RNA interference

The PBMCs from whole blood samples of healthy donors were isolated using Ficoll-PaqueTM PLUS reagent according to the manufacturer's protocol (GE, Sweden). For interference with human SETD2, human $CD4^+$ T cells were purified from PBMCs. $CD4^+$ T cells were then transfected with 100 nM of siRNA-SETD2 using P3 Primary Cell 4D-Nucleofector X Kit reagent (Lonza) following the manufacturer's instructions. We then changed to Th17 or iTreg cell differentiation medium after 24 hours of transfection. siRNA sequences are specific for SETD2 and listed in Supplementary Table 1. The healthy donor samples were obtained with written informed consent and with the approval of the Scientific Investigation Board of Naval Medical University and the Institutional Review Board (IRB) of Changhai Hospital.

Lentiviral infection

The Lpcat4 and Setd2 lentiviral were obtained from Genechem (Genechem, China). $CD4^+$ T cells were stimulated with α-CD3/CD28 for 24 hours. The activated $CD4^+$ T cells were pun infected for 90 min at 1500 rpm and polybrene (10 μg/ml) and incubated at 37°C for 24 hours, and then were changed to Th17 or iTreg cell differentiation medium.

Real-time quantitative PCR

Total RNA was extracted with TRIzol reagent according to the manufacturer's instructions. cDNA was synthesized from RNA with Oligo(dT) primer and M-MLV (RNase H-) Kit (Takara). mRNA expression was analyzed by QuantStudio 6 or 7 Flex (Thermo, Applied Biosystems) using Premix Ex Taq II Kit (Takara) and normalized to

β-Actin. The primer sequences used are listed in Supplementary Table 1.

ChIP-qPCR Assay

Naïve $CD4^+$ T, Th17, and iTreg cells were harvested and manipulated according to the Chromatin Immunoprecipitation (ChIP) Assay Kit (Millipore) protocol. ChIP assays were performed with anti-H3K36me3 antibody (Abcam), anti-Rpb1 CTD antibody, anti-HIF-1α antibody, or normal rabbit IgG antibody (Cell Signaling Technology). The primers for qPCR are listed as follows: *Lpcat4*, forward: GGG CAT GTC TCT AAG GGT GT, reverse: AGA AAG GCA GGG TTG TGA GT; *Rorc*, forward: CCC CAC ACC AGA AGT AT, reverse: CCT CTG TGT GTG TGT GTG TG; *Il17*, forward: AGC TCC CAA GAA GTC ATG CT, reverse: TAC GTC AAG AGT GGG TTG GG.

Immunoblot analysis

The immunoblot assay was performed as described previously (1). Naïve $CD4^+$ T, Th17, and iTreg cells were lysed in 1× Cell Lysis Buffer (Cell Signaling Technology) to obtain whole-cell lysates, and protein concentrations were detected with bicinchoninic acid assay (Thermo Fisher Scientific). Equalized extracts were used for immunoblot analysis. Antibodies were used for immunoblot assay as follows: anti-Setd2 and anti-Lpcat4 were obtained from ABclonal Technology. Anti-Vinculin, anti-ROR gamma, anti-Foxp3, anti-PERK, anti-Histone H3 (tri methyl K36), anti-Histone H3 were obtained from Abcam. Anti-β-Actin, anti-HIF-1α, anti-Phospho-eIF2α (Ser51), anti-eIF2α were obtained from Cell Signaling Technology.

Confocal microscopy

Differentiated Th17 cells were harvested and incubated with 1 μM ER Tracker

(Molecular Probes) for 20 minutes at 37 °C, and finally stained with DAPI for 5 min at room temperature. Cells were transferred to a microscope slide by cytocentrifugation for confocal microscopy. Images were captured with a laser scanning confocal microscope (Leica TCS SP8) and analyzed by the LAS X software version 2.0.2.15022.

Phosphatidylcholine dissolution method

Phospholipid dissolution was performed as described previously (2). The phosphatidylcholines PC(16:0, 18:1) and PC(16:0, 18:2) (Avanti Polar Lipids, Inc.) were suspended in RPMI1640 medium containing 10% FBS, gently mixed, and sonicated at room temperature for 15 min for complete dissolution followed by supplementation with cell culture medium.

Lipidomics profiles

1×10^7 differentiated Th17 cells were harvested and frozen immediately. The frozen samples were mixed with lipid extraction solution and an internal standards mixture by vortex for 2 minutes. We emulsified the mixed sample, sonicated for 5 minutes, mixed with 500 μl water, and vortexed for 1 minute. The supernatant was collected and dried with nitrogen and re-dissolved with 100 μl of mobile phase B. Ultra-Performance Liquid Chromatography (UPLC) and Tandem Mass Spectrometry (MS/MS) analysis were then carried out (UPLC, ExionLCTMAD, https://sciex.com.cn/; MS/MS, QTRAP R 6500+, https://sciex.com.cn/). The lipid composition was detected by MetWare (http://www.metware.cn/) based on the AB Sciex QTRAP 6500.

Psoriasis-like mouse model

The shaved backs of C57BL/6J (6-8 weeks of age) mice were treated with a daily dose of 62.5 mg of imiquimod cream (Sichuan Med-shine Pharmaceutical) for 7 consecutive days. Control mice were treated with the same dose of glycerol cream.

Active and passive induction of EAE

The induction of active EAE or adoptive transfer EAE was described previously (1). In brief, female mice at the age of 10 to 12 weeks were immunized with 2 mg/ml MOG_{35-55} (Sangon) in Freund's Complete Adjuvant (Thermo Fisher Scientific), then injected 2 μg/ml pertussis toxin (Gibco) intraperitoneally on day 0 and day 2. Solvent or PC(16:0, 18:2) (Avanti Polar Lipids, Inc.) were suspended in PBS (30mg kg^{-1}) was intraperitoneally injected every other day from day 4 to day 18.

For adoptive transfer EAE, naïve $CD4^+$ T cells were purified from the spleens of WT and *Setd2*-CD4 cre mice, and intravenously transferred into *Rag2*$^{-/-}$ mice at 2.5 $\times 10^6$ cells per mouse, and then induced EAE in these *Rag2*$^{-/-}$ mice. Clinical scores were evaluated according to the following standards: 0, normal; 1, limp tail; 2, limp tail, impaired righting reflex, and paresis of one limb; 3, hind-limb paralysis; 4, hind-limb and fore-limb paralysis; and 5, moribund.

For histological scoring, spinal cord sections were scored as follows: 0, no infiltration (<50 cells); 1, mild infiltration (50-100 cells); 2, moderate infiltration (100-150 cells); 3, severe infiltration (150-200 cells); 4, massive infiltration (>200 cells) (3).

SI References

1. Chen, Y. L., J. Zhang, XM. Zhu, H. Wang, YJ. Li, ZQ. Liu, YF. Liu, S. Liu, SX. Li, N. Chen, K. Cao, X., lncRNA-GM targets Foxo1 to promote T cell-mediated autoimmunity. *Sci Adv.* **8**, eabn9181 (2022).
2. Thurmer, M. *et al.*, PI(18:1/18:1) is a SCD1-derived lipokine that limits stress signaling. *Nat Commun*. **13**, 2982 (2022).
3. Aso, K. *et al.*, Itaconate ameliorates autoimmunity by modulating T cell imbalance via metabolic and epigenetic reprogramming. *Nature Communications*. **14**, 984 (2023).

Supplementary Figures

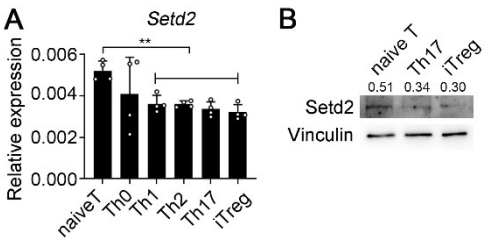

Fig. S1. The expression of Setd2 in T cells.

(*A*) qPCR analysis of *Setd2* mRNA expression in T cells (n=4). (*B*) Immunoblot analysis of Setd2 levels in naïve $CD4^+$ T, Th17, and iTreg cells. Data show one of three independent experiments. Results are presented as means ± SD (*A*). $^{**}P < 0.01$.

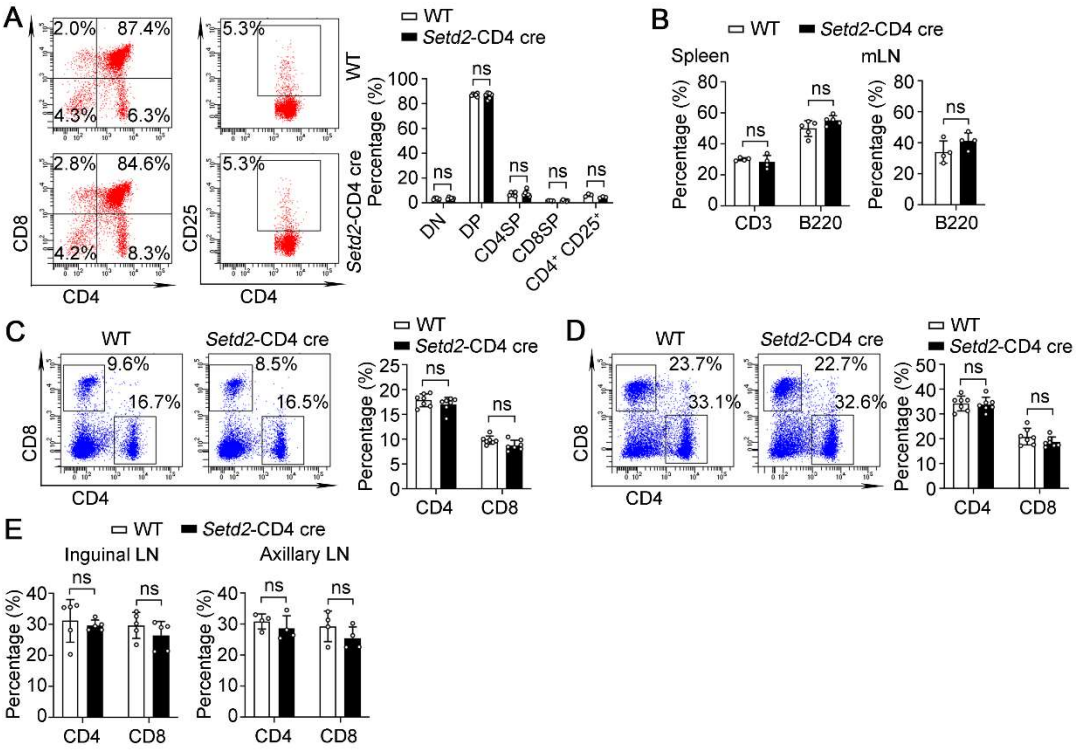

Fig. S2. Setd2 does not affect the development of T cells.

(*A*) Flow cytometric analysis and quantification of DN ($CD4^-$ $CD8^-$), DP ($CD4^+$ $CD8^+$), CD4SP ($CD4^+$ $CD8^-$), CD8SP ($CD4^-$ $CD8^+$), and nTreg ($CD4^+$ $CD25^+$) thymocytes (n=3 to 6). (*B*) Frequency of $CD3^+$ and $B220^+$ cells in the spleen and mLN from WT and *Setd2*-CD4 cre mice, assessed by flow cytometry (n=4 to 5). (*C* to *E*) Flow cytometric analysis and quantification of $CD4^+$ and $CD8^+$ cells in the spleen (*C*), mLN (*D*), inguinal LN and axillary LN (*E*) (n=4 to 7). Data show one of three independent experiments. Results are presented as means ± SD (*A*-*E*). ns, not significant. mLN, mesenteric lymph node.

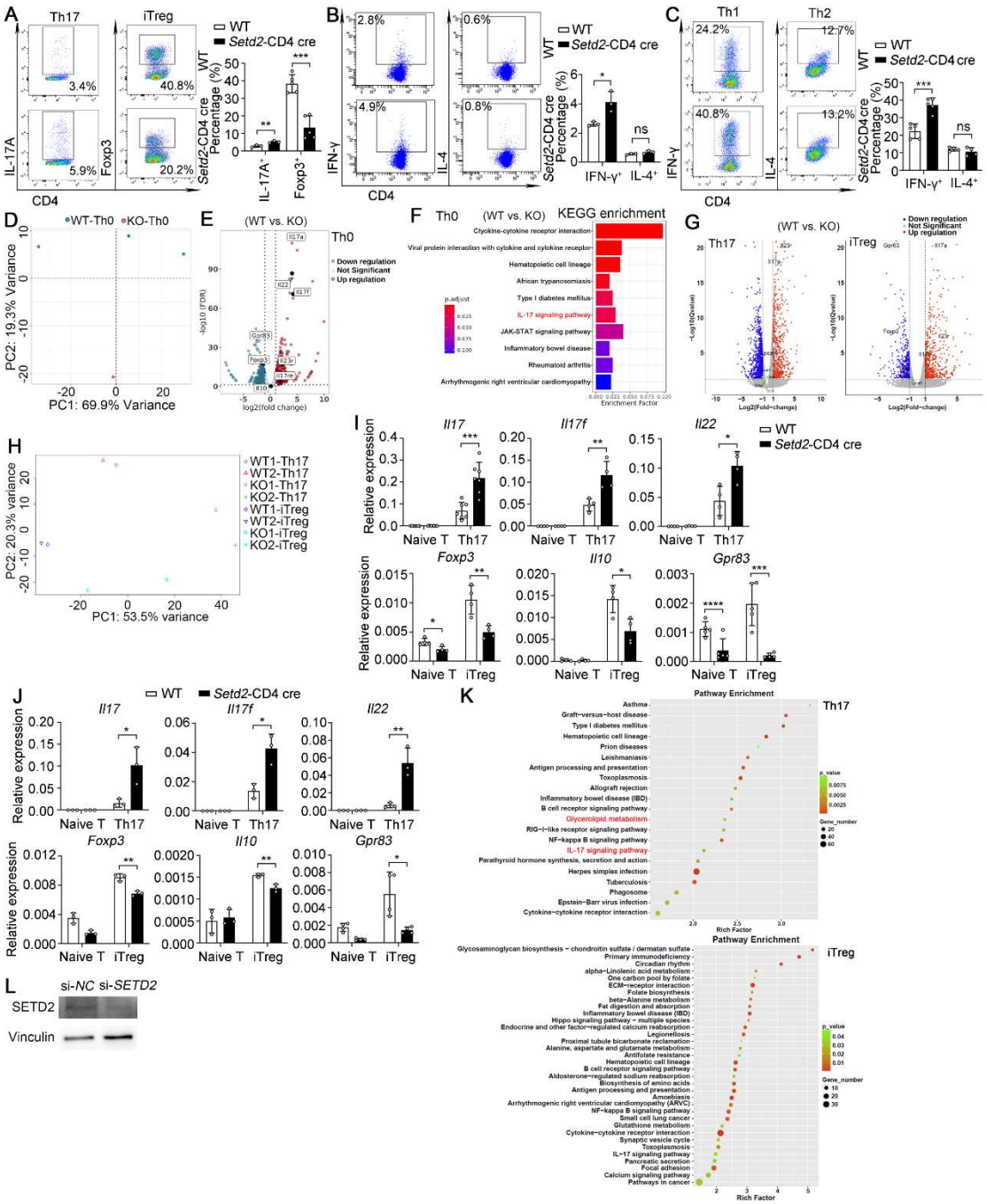

Fig. S3. Deficiency of *Setd2* promotes Th17 and inhibits iTreg cell polarization.

(*A*) Analysis and quantification of differentiated Th17 and iTreg cells from mesenteric lymph node (mLN) by flow cytometry (n=3 to 5). (*B* and *C*) Flow cytometric analysis IFN-γ^+ and IL-4^+ cells in the spleen (*B*) and differentiated Th1 and Th2 cells (*C*) (n=3 to 6). (*D*) Principle component analysis of global gene expression from RNA-seq in

Th0 cells. (*E*) Volcano plot of differential gene expression in Th0 cells. (*F*) KEGG pathway analysis of RNA-seq data from Th0 cells ($P < 0.05$). (*G*) Volcano plot of differential gene expression in Th17 and iTreg cells. (*H*) Principle component analysis of global gene expression from RNA-seq in Th17 and iTreg cells. (*I* and *J*) qPCR analysis of Th17-related genes and iTreg-related genes from spleen (*I*) and mLN (*J*) (n=3 to 7). (*K*) KEGG pathway analysis of RNA-seq data from Th17 ($P < 0.01$) and iTreg cells ($P < 0.05$). (*L*) Immunoblot analysis of SETD2 levels transfected with *SETD2* siRNA (si-*SETD2*) or control siRNA (si-*NC*) in naïve $CD4^+$ T. Data show one of three independent experiments. Results are presented as means ± SD (*A-C, I* and *J*). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. ns, not significant.

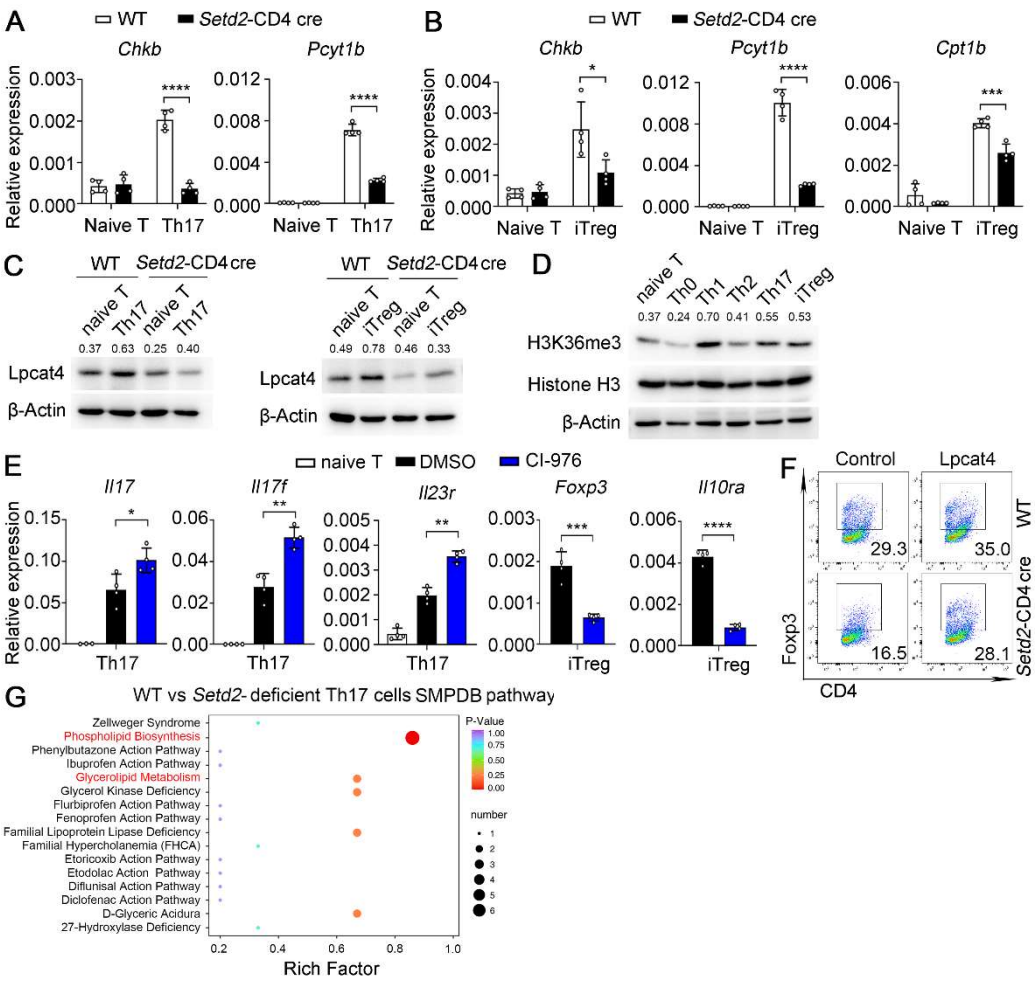

Fig. S4. Deficiency of *Setd2* promotes Th17 and inhibits iTreg cell differentiation by regulating phospholipid synthesis.

(*A* and *B*) qPCR analysis of *Chkb*, *Pcyt1b,* and *Cpt1b* mRNA expression in Th17 and iTreg cells (n=4). (*C*) Protein levels of *Lpcat4* expression in T cells. (*D*) Immunoblot analysis of H3K36me3 levels in naïve $CD4^+$ T, Th0 (αCD3/CD28), Th1, Th2, Th17, and iTreg cells. (*E*) qPCR analysis of *Il17*, *Il17f, Il23r, Foxp3* and *Il10ra* mRNA levels in naïve $CD4^+$ T, Th17 and iTreg cells after stimulation with DMSO or CI-976 (10 μM) (n=4). (*F*) Flow cytometric analysis of $Foxp3^+$ cells transfected with control and Lpcat4 expressing lentivirus in iTreg cells. (*G*) SMPDB pathway analysis of

differential lipid metabolites in WT and *Setd2*-deficient Th17 cells. Data show one of three independent experiments. Results are presented as means ± SD (*A, B* and *E*). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. SMPDB, Small Molecule Pathway Database.

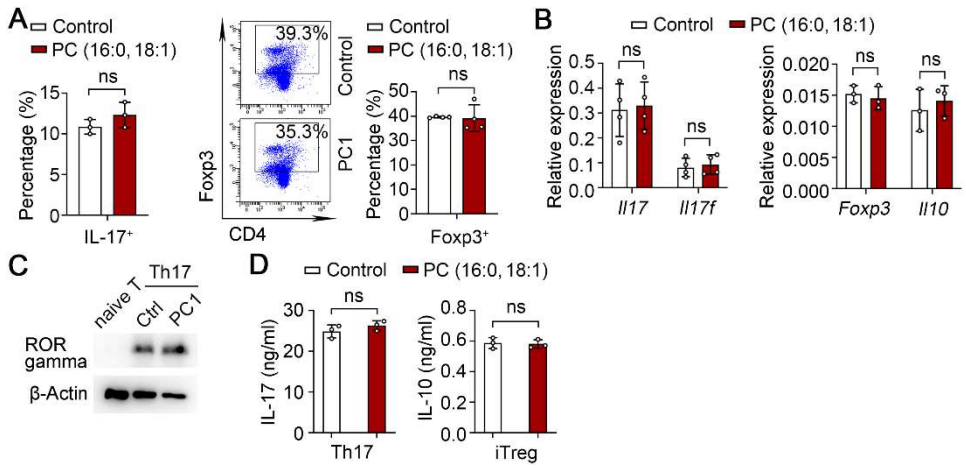

Fig. S5. Lpcat4-mediated PC(16:0, 18:1) does not affect Th17 or iTreg cell differentiation.

(*A*) Flow cytometric analysis and quantification of IL-17A$^+$ in Th17 (left) and Foxp3$^+$ in iTreg (right) cells after treatment with solvent control or PC(16:0, 18:1) (PC1, 50 μM) (n=3 to 4). (*B*) qPCR analysis of *Il17*, *Il17f*, *Foxp3,* and *Il10* mRNA expression in Th17 (left) and iTreg (right) cells after stimulation with control or PC(16:0, 18:1) (n=3 to 4). (*C*) Immunoblot analysis of RORγt levels in Th17 cells after treatment with control or PC(16:0, 18:1). (*D*) CBA analysis of IL-17 in Th17 and IL-10 in iTreg cells after treatment with control or PC(16:0, 18:1) (n=3). Data show one of three independent experiments. Results are presented as means ± SD (*A*, *B*, and *D*). ns, not significant. PC1 referred to PC(16:0, 18:1).

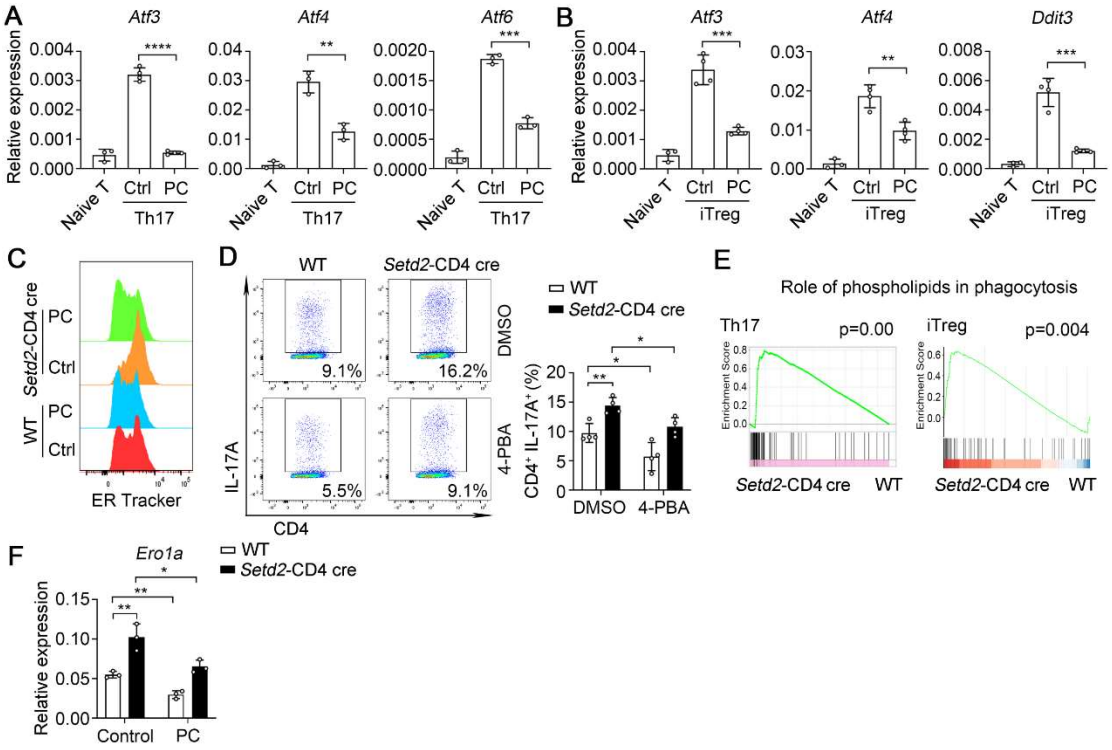

Fig. S6. Setd2 promotes PC(16:0, 18:2) production to inhibit ER stress and oxidative stress.

(*A* and *B*) mRNA expression levels of ER stress markers in Th17 (*A*) and iTreg (*B*) cells stimulated with control (Ctrl) or PC(16:0, 18:2) (PC, 50 μM) (n=3 to 4). (*C*) Analysis of ER tracker MFI in iTreg cells treated with control (Ctrl) or PC(16:0, 18:2). (*D*) Flow cytometric analysis and quantification of IL-17A$^+$ in Th17 cells after treatment with DMSO or 4-PBA (1mM) (n=4). (*E*) GSEA analysis of enrichment in phospholipids in phagocytosis pathway from Th17 and iTreg cells. (*F*) qPCR analysis of *Ero1a* mRNA levels in Th17 cells after stimulation with control or PC (n=3). Data show one of three independent experiments. Results are presented as means ± SD (*A*, *B*, *D*, and *F*). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. 4-PBA, 4-phenylbutyric acid. MFI, mean fluorescence intensity.

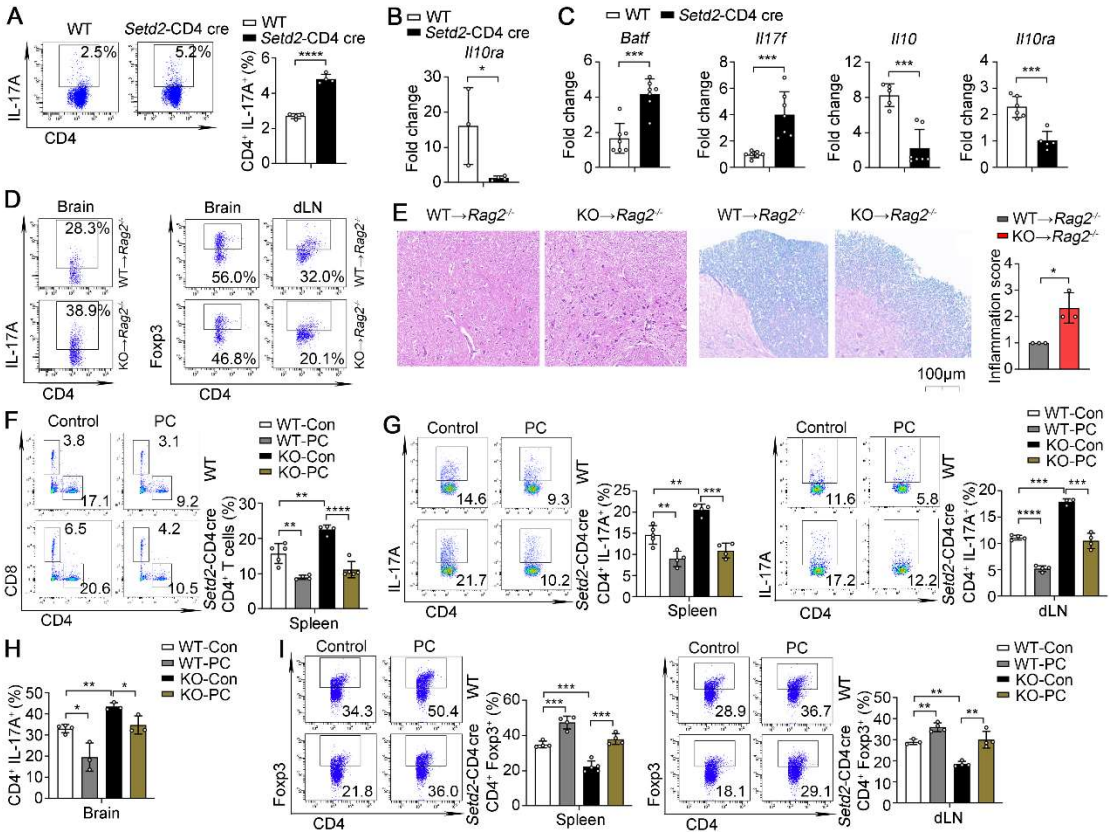

Fig. S7. Deficiency of *Setd2* aggravates the pathogenesis of autoimmune diseases.

(*A* to *C*) All mice were subjected to induction of experimental autoimmune encephalomyelitis (EAE). (*A*) Frequency and quantification of IL-17A$^+$ cells in the mesenteric lymph node of WT and *Setd2*-CD4 cre mice (n=4). (*B*) mRNA expression of *Il10ra* in the spleen, assessed by qPCR (n=3 to 4). (*C*) mRNA expression of *Batf*, *Il17f, Il10*, and *Il10ra* in the brain (n=5 to 7). (*D* and *E*) Adoptive transfer of WT and *Setd2*-deficient CD4$^+$ T cells into *Rag2*$^{-/-}$ host mice and induced EAE. (*D*) Frequency of IL-17A$^+$ and Foxp3$^+$ T cells in the brain and dLN of *Rag2*$^{-/-}$ recipient mice. (*E*) Representative histology of the spinal cord stained with hematoxylin and eosin and luxol fast blue of *Rag2*$^{-/-}$ recipient mice after EAE induction (day 15). And inflammation scores of spinal cords are shown. (*F* to *I*) EAE mice were

intraperitoneally injected with 30mg kg^{-1} PC(16:0,18:2) or solvent control every other day from day 4 to day 18. (*F*) Frequency of $CD4^+$ and $CD8^+$ T cells in EAE mice treated with PC(16:0,18:2) or control (n=3 to 5). (*G* and *H*) Frequency of IL-$17A^+$ cells in the spleen, dLN (*G*), and brain (*H*) from EAE mice treated with PC(16:0,18:2) or control. (*I*) Frequency of $Foxp3^+$ cells in the spleen and dLN from EAE mice treated with PC(16:0,18:2) or control. Data show one of three independent experiments. Results are presented as means ± SD (*A*-C, and *E*-*I*). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

Supplementary Table 1. qPCR primers and siRNA sequences used in this study

Gene primers and siRNA	Sequences (5'→3')
mIl17, forward	CTCAAAGCTCAGCGTGTCCAAACA
mIl17, reverse	TATCAGGGTCTTCATTGCGGTGGA
mIl17f, forward	CAGGAAGACAGCACCATGAA
mIl17f, reverse	TCTTCTCCAACCTGAAGGAATTAG
mIl23r, forward	CACTGCCGACCAAGGAATCT
mIl23r, reverse	GCATGAGGTTCCGAAAAGCC
mIl22, forward	CAACTGTTGACACTTGTGCGA
mIl22, reverse	ACAGGGCAATGAGAAGCAGG
mCcr6, forward	CAGCTTGGAGCAGAATAGCAA
mCcr6, reverse	GGAGAGCAGAGGTGAAGCAATA
mFoxp3, forward	GCGAAAGTGGCAGAGAGGTA
mFoxp3, reverse	GAGGAGCTGCTGAGATGTGA
mGpr83, forward	CGCCCTTCACTTTGGTCATC
mGpr83, reverse	CAGAGGGAGCGCACAATGTC
mIl10ra, forward	TGCATACGGGACAGAACTGC
mIl10ra, reverse	CAGGACAATGCCTGAGCCTT
mIl10, forward	GCTCCAAGACCAAGGTGTCT
mIl10, reverse	CGGAGAGAGGTACAAACGAGG
mChkb, forward	GCGTGATGTTCGCCATTCTC
mChkb, reverse	TCCATACCATGGAAACGGGC
mPcyt1b, forward	AATGAACAACAGCCCAGAGGG
mPcyt1b, reverse	AAGGCAGCTTGATATGCCCAG
mCpt1b, forward	CCTACCACGGGTGGATGTTC
mCpt1b, reverse	ATAAGGGCCGCACAGAATCC
mLpcat4, forward	GACCCCACCCTTTATGCCAA
mLpcat4, reverse	TGGCTGATCATTCGACTCCG
mAtf4, forward	CCTATAAAGGCTTGCGGCCA
mAtf4, reverse	GTCCGTTACAGCAACACTGC
mAtf6, forward	CATGTGTGGAAGAAATGCAGGA
mAtf6, reverse	CCTCTTTAGGCTTCCATTCCCA
mDnajb9, forward	CTCGGATGCCAATAGTCGGA
mDnajb9, reverse	TAGAACCATCCTGGCGTGTG
mAtf3, forward	CCTTATCATCCCGGCCAGTC
mAtf3, reverse	CTGGCCATTGGACAACCTCA
mDdit3, forward	CCTGAGGAGAGAGTGTTCCAG
mDdit3, reverse	CTTCCTCTTCGTTTCCTGGGG
mEro1a, forward	CTACACAGGCTACAAGGGGC

mEro1a, reverse	GCACATTAATGCTTGCGTGC
mHif1a, forward	AGGATGAGTTCTGAACGTCGAAA
mHif1a, reverse	CTGTCTAGACCACCGGCATC
mSetd2, forward	AATGGACAGCTGAGGGTTGG
mSetd2, reverse	GGCTGACCCACAGAAACACT
hSETD2, forward	CCCGACCCCTGAAGAAGAAG
hSETD2, reverse	GCCAAGTGCAGTGAGAAACC
hRORC, forward	CCAAGGCTCAGTCATGAGAAC
hRORC, reverse	AGGAGTAGGCCGCGTTACA
hIL17F, forward	GAAAACCAGCGCGTTTCCAT
hIL17F, reverse	GCAGCCCAAGTTCCTACACT
hIL23R, forward	GCCTGGCTCTGAAGTGGAAT
hIL23R, reverse	CCTCCATGACACCAGCTGAA
hIL21, forward	TTGCTTCTTAGTTACTCACGGT
hIL21, reverse	TGTCCAACTGCAAGTTAGATCCT
hBATF, forward	GAAAACCAGCGCGTTTCCAT
hBATF, reverse	GCAGCCCAAGTTCCTACACT
hFOXP3, forward	GGCCACATTTCATGCACCAG
hFOXP3, reverse	GCTCCCTGGACACCCATTC
SETD2 siRNA-1 sense	GCUCCAUCAAAUCGAUUAATT
SETD2 siRNA-1 antisense	UUAAUCGAUUUGAUGGAGCTT
SETD2 siRNA-2 sense	GGUGUAACUUAUGCAUUAATT
SETD2 siRNA-2 antisense	UUAAUGCAUAAGUUACACCTT
SETD2 siRNA-3 sense	GCCCAUGGAAGAAACAAGUTT
SETD2 siRNA-3 antisense	ACUUGUUUCUUCCAUGGGCTT
Lpcat4 siRNA-1 sense	GGCGUACUCAAAGUUCUCUTT
Lpcat4 siRNA-1 antisense	AGAGAACUUUGAGUACGCCTT
Lpcat4 siRNA-2 sense	CCCAAUCCGAGUACUUCUATT
Lpcat4 siRNA-2 antisense	UAGAAGUACUCGGAUUGGGTT
Lpcat4 siRNA-3 sense	GUCGAAUGAUCAGCCAAGATT
Lpcat4 siRNA-3 antisense	UCUUGGCUGAUCAUUCGACTT