PNAS

Supporting Information for

The PRAK-NRF2 axis promotes the differentiation of Th17 cells by mediating the redox homeostasis and glycolysis

Ziheng Zhao1,#,Yan Wang2,#, Yuhan Gao1,3, Yurong Ju1, Ye Zhao1, Zhaofei Wu1, Shuaixin Gao4, Boyang Zhang1, Xuewen Pang1, Yu Zhang1,5,* and Wei Wang1*
1 Department of Immunology, School of Basic Medical Sciences, Peking University. NHC Key Laboratory of Medical Immunology (Peking University), Beijing, China.
2 First Clinical Medical College, Shanxi Medical University, Taiyuan, 030001, Shanxi, China
3 Departments of Blood Transfusion, Peking University of People's Hospital, Beijing, China.
4 Center for Precision Medicine Multi-Omics Research, Peking University Health Science Center, Beijing, China
5 Institute of Biological Sciences, Jinzhou Medical University, Jinzhou, Liaoning, China.
#These authors contribute equally to this work.

* Yu Zhang and Wei Wang.
Email: wangwei83427@bjmu.edu.cn and zhangyu007@hsc.pku.edu.cn

This PDF file includes:

Extended methods

Mice

PRAKflox/flox mice were generated by Shanghai Model Organisms Center (China) through flank the exon 5 of *PRAK* gene with loxp sequence, deletion is ready to be detected by PCR (Forward primer:AGGCAGCAAGCAAGGCACC, reverse primer:CTGAAGCATCAAACACTGACCTAAA. Mutant: 453bp; Wild type:399bp). CD4Cre mice was purchased from Jacksonlab. To generate mice with selective PRAK deficiency in T cells, PRAKflox/flox mice were crossed with CD4Cre mice, both on C57BL/6J background. Mice were kept in the specific-pathogen-free conditions animal facility of the Peking University Health Science Center. The experimental procedures on use and care of animals were approved by the Ethics Committee of Peking University Health Science Center.

Flow cytometry

For surface staining, cells were incubated for 30 min at 4°C with fluorescent-labeled monoclonal antibodies specific for mouse CD4, CD45, CD8 (BioLegend). After the surface staining, cells were fixed and permeabilized with Foxp3 / Transcription Factor Fixation/Permeabilization Concentrate and Diluent Kit (Thermo Fisher) according to the manufacturer's instructions, and stained with fluorescent-labeled monoclonal antibodies specific for mouse IL-4, IL-5, IFN-γ, GranzymeB, IL-17A, Foxp3, TNF-α, Ki67 (BioLegend). Especially for the analysis of IFN-γ, GranzymeB, IL-17A and TNF-α, cells were harvested and stimulated with 100ng/ml PMA+ 1μg/ml ionomycin +10μg/ml BFA in RPMI1640 medium at 37℃ for 6h and stained as described above. For the analysis of IL-4 and IL-5, cells were stimulated with 2μg/ml anti-mouse-CD3 and 10μg/ml monesin in RPMI1640 medium at 37℃ for 6h and stained as described above.To assay ROS production, lymphocytes were incubated with 10 μM 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA, Invitrogen) for 1 h in RPMI 1640 complete medium at 37°C before surface staining. To assay glucose intake, differentiated Th17 cells were incubated with 10 μg/ml 2-NBDG (APExBIO) for 15 min in RPMI 1640 medium at 37°C and washed with PBS. For lipid metabolism assay, differentiated Th17 cells were washed and resuspended in 500 μl of BODIPY 493/503 (for neutral lipid content, Life Technologies) or C1-BODIPY 500/510 C16 (for lipid intake, Life Technologies) at a final concentration of 0.5 μM in RPMI 1640 medium at 37°C for 15min and washed with PBS. The MOG_{35-55} tetramer staining was operated as manufacturers' instructions. Flow cytometry data were collected on FACSCanto (BD Biosciences) and analyzed using Kaluza or Flowjo software.

Cell purification and culture

Naïve CD4+ CD62L+ T cells were enriched with the Naive CD4+ T Cell Isolation Kit (cat: 130-104-453, MiltenyiBiotec).CD3+ T cells with CD3e MicroBead Kit mouse (cat: 130-094-973, MiltenyiBiotec). CD19+ B cells with CD19 MicroBeads mouse (cat: 130-052-201, MiltenyiBiotec). Naïve CD4+ T cells were suspended in RPMI 1640(cat: C11875500BT, Gibco) supplemented with 10% heat-inactivated fetal bovine serum and 50μg/ml gentamicin(cat: L1312,Solarbio) at 1×10^6/ml in 96-well plates and induced as follows.Th17 conditions include anti-mouse-CD3 (2μg/mL, BioLegend), soluble anti-mouse-CD28 (1μg/mL, BioLegend), TGF-b1 (0.5 ng/mL, R&D), mouse IL-6 (30 ng/ml, R&D), Anti-mouse-IL-4(10μg/ml, BioLegend), Anti-mouse-IFN-γ(2μg/ml, BioLegend). Th1 conditions include anti-mouse-CD3 (2μg/mL, Biolegend), soluble anti-mouse-CD28 (1μg/mL, BioLegend), mouse IL-12 (100 ng/ml, Peprotech). Th2 conditions include anti-mouse-CD3 (2μg/mL, Biolegend), soluble anti-mouse-CD28 (1μg/mL, BioLegend), mouse IL-4 (30 ng/ml, Peprotech),anti-mouse-IFN-γ (2μg/ml, BioLegend). Treg conditions include anti-mouse-CD3 (2μg/mL, Biolegend), soluble anti-mouse-CD28 (1μg/mL, BioLegend), mouse IL-2 (10 ng/ml, Peprotech), TGF-β1 (10ng/ml, BioLegend).3 days later, the differentiation efficacy was evaluated via flow cytometry.

MC38 Tumor Model

In order to construct tumor-bearing mouse models of MC38 cells, we cultured MC38 cells with DMEM culture media (Gibco, C11965500BT) into logarithmic phase, and suspend MC38 cells into 1×10^6/100μl with PBS. 1×10^6 or 3×10^6(for survival analysis) MC38 cells were injected subcutaneously into each mouse. The calculation formula of tumor volume is

$V = \pi \times width^2 \times length/2$. For CD4 depletion and IL-17A neutralization, mice were intraperitoneally injected with a single dose of 500μg anti-CD4 (Selleck, clone number GK1.5), anti-IL-17A (Selleck, clone number 17F3), or isotype control (IgG2b and IgG1, respectively) antibodies, one day before the inoculation. For NAC treatment, 67mg/kg NAC dissolved in PBS or isovolumetric PBS were injected intraperitoneally once a day. For the tumor-infiltrating lymphocyte analysis, tumors were first cut into pieces and digested with 1mg/ml collagenase IV(Solarbio) and 100μg/ml DNAseI (Roche) in 1640 for 1 hours in room temperature, and grinded and enriched with Mouse Tumor infiltrating Tissue lymphocyte Isolation kit (Solarbio) according to manufacturers' instructions for downstream flow cytometer analysis.

EAE Model

Active EAE were induced by subcutaneous immunization at the tail base with 150μg/mice MOG_{35-55} which was emulsified in 100μL of complete Freund's adjuvant on day 1 and day 7, and then followed by i.p. injection of 500μg/mice PTX dissolved in PBS on day 2 and day 8. The disease was scored as described in(1).

Western Blotting

The cross link of PKM2 and western blot was performed as described in (2). The separation of nuclear and cytoplasmic fractions was performed using the PARIS Kit (cat:AM1921, Thermofisher) according to manufacturers' instructions. Quantification of protein expression was performed using ImageJ software (version 1.50i). Briefly, cells were lysed and the cell lysate was resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinyli-dene diuoride membrane (Millipore, Boston, MA, USA). The membrane was probed with antibodies specific for Y105P STAT3, STAT3, Histone-H3, NRF2, HO1, Prdx2, Keap1, FLAG, HA, GSK3, HK2, Glut1, PKM2, Enolase1, Vinculin (Cell Signaling Technology), PRAK, β-actin (Proteintech). Following incubation with HRP labeled secondary antibodies (Invitrogen), the immunoreactive bands were revealed and analyzed using the Super Signal chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA).

RNA-seq and analysis

Both WT and KO Th17 cells were collected after 3 days polarization and the total RNA was extracted with Trizol (Life Technologies) according to manufacturer's instructions. Further processing was completed by using a BGISEQ platform (Shenzhen Huada Gene Science and Technology Service Co., Ltd., Shenzhen, China). The BGISEQ platform was applied to determine differentially expressed genes (DEGs). Genes with a fold-change >1.2 and an adjusted p-value < 0.05 were defined as DEGs. DEGs were used for GO enrichment analysis on the BGISEQ platform. All the glycolysis genes were used to make the GSEA on the BGISEQ platform. The R package pheatmap was applied to make heatmap based on the DEGs.

Patient survival analysis

The survival analysis was performed on Gene Expression Profiling Interactive Analysis (GEPIA,http://gepia.cancer-pku.cn/index.html)(3). Analysis was performed restricted to colon cancer patients' data from TCGA database.

Reagents

Phorbol-myristate-acetate (PMA), ionomycin, N-Acetyl-L-cysteine (NAC) and Complete Freund's adjuvant were purchased from Sigma-Aldrich. TEPP-46, MG132 were purchased from Selleck. Lactacystin was purchased from Abcam. DSS was purchased from Thermo Fisher. PRAK inhibitor GLPG0259 was synthesized by Wuxi Pharma (China). $MOG_{35-55}$was synthesized by CHINESE PEPTIDE (China). pertussis toxin (PTX) was bought from List Biological laboratories. BFA and monesin were bought from Biolegend. Polyethylenimine Linear, MW 40000(PEI 40000) was purchased from Yeasen. Puromycin was purchased from Solarbio. T-Select I-Ab MOG35-55 tetramer (APC) was purchased from MBL Beijing Biotech CO.

MS

Equal quality of WT flag-tagged NRF2 plasmid was transduced to WT and PRAK KO HEK293T cells for 24h, then the cells were harvested and lysed with Co-IP lysis (20mM Tris·HCl, pH= 7.5, 150mM NaCl,1% Triton X-100) in 4℃ for 30min. The lysis was heated in 95℃ for 10min and disrupted ultrasonically, then centrifuged and did the IP experiment with flag antibody using the supernatants. The IP enriched Flag-tagged NRF2 protein sample was purified with SDS-PAGE and next coomassie blue staining. The band at ~130kDa was cut and analyzed in Center for Precision Medicine Multi-Omics Research, Peking University Health Science Center.

Glucose measurement
The glucose concentrationsin the supernatants were quantified by Glucose Detection Kit (O-toluidine method) bought from Beyotime and operating according to manufacturers' instructions.

Quantitative Real-Time RT-PCR
Total RNA was isolated from T cells with the use of Trizol reagent (Invitrogen) according to manufacturer's instructions. cDNAs were synthesized with Superscript reverse transcriptase and oligo(dT) primers (Invitrogen) and analyzed with an iCycler Optical System and an iQ SYBR green real-time PCR kit (Roche). The data were normalized to a β-actin control.

Generation of PRAK-KO HEK293T cells
Human PRAK-gRNA (ATCAATTGGACTCAGAAGCT) was cloned into lenti-CRISPR-v2 backbone (52961, addgene). The blank backbone or gRNA plasmid was transduced into HEK293T cells via PEI. Transfected HEK293T cells were screened with 5μg/ml puromycin for 1 week, and the KO efficacy was verified by PCR test. Targeted PRAK-KO or WT single clone was picked and amplified for the next experiments.

Seahorse
The murine naïve CD4+ T cells were separated and polarized into Th17 cells as described above for 24h and plated into a seahorse XF24 microwell plate (cat:100882-004, Agilent) at 1×10^6/well. further processing was done by Beijing Pangu Bioscience Technology Co.,LTD (China)

Ubiquitin measurement
Flag tagged NRF2 plasmids were transduced to WT and PRAK KO HEK293T cell lines. 24h later, the OE-HEK293T was treated with 20μM MG132 for another 12h, and the cells were harvested, lysed and IP using Flag antibodies for the next western blot.

Half-life time measurement
Flag tagged WT or mutated NRF2 plasmids were transduced to WT and PRAK KO HEK293T cell lines. 24h later, the OE-HEK293T was treated with 200μM cycloheximide (CHX) and harvested every one or two hours for next 4 hours for western blot analysis.

ELISA
Serum or supernatants was collected and the IL-17A cytokine level was measured by ELISA Kit (invitrogene) according to manufacturers' instructions.

Immunohistochemical staining (IHC)
The tissues were extracted and fixed with formalin. The next procedure was accomplished by Servicebio (Wuhan, China). Serial sections were used to identify multiple positive cells.

Statistical analysis
All Statistical analyses and all statistical graphs and survival curves were generated using GraphPad Prism 8 (USA, GraphPad Software). When indicated, the statistical significance was determined by Student's t test ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

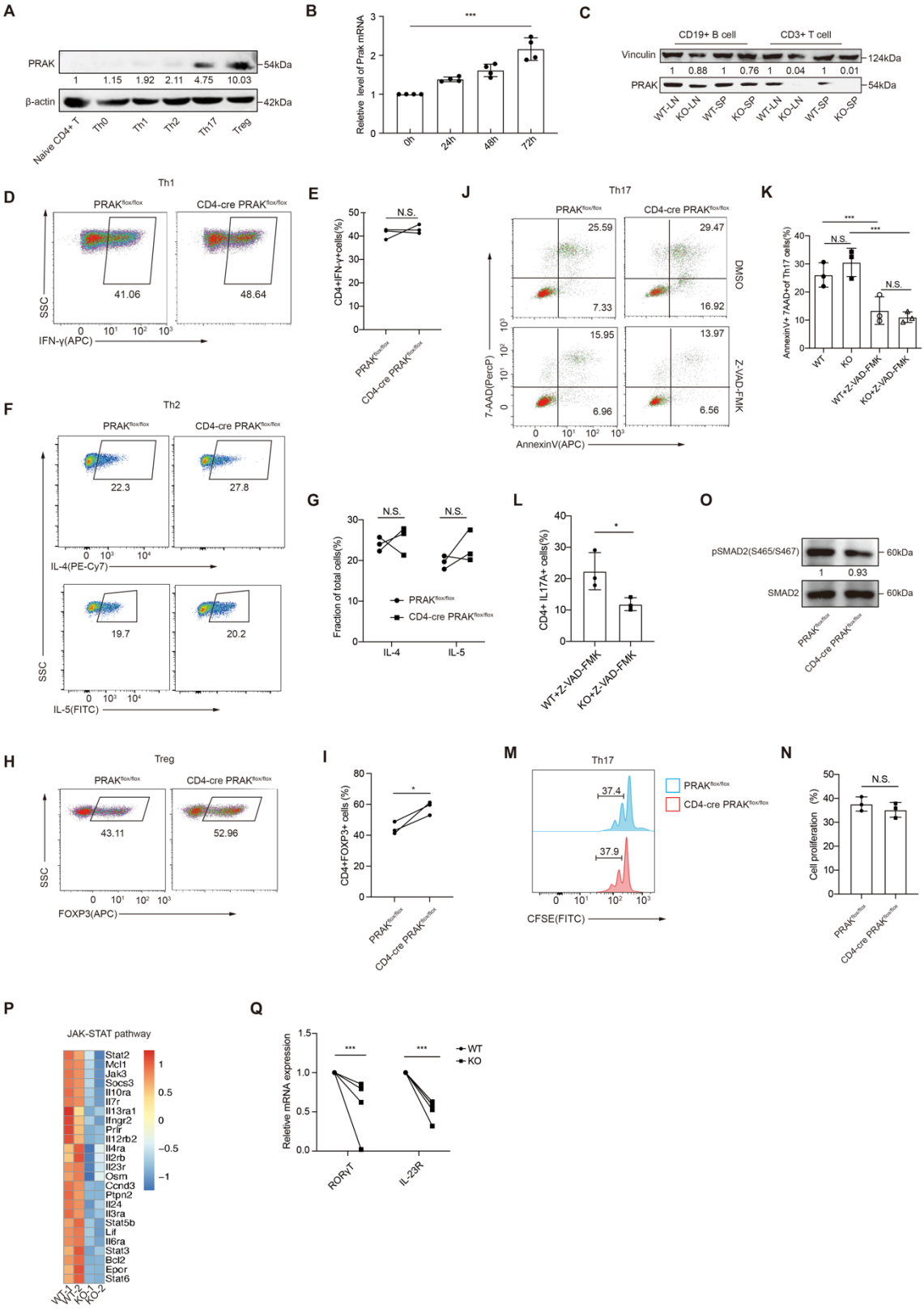


Fig. S1.The critical role of PRAK in the differentiation of Th17 cells.(A) Western blot analysis of PRAK expression in naïve CD4+ T cells, and Th0(without any cytokines), Th1(IL-12), Th2(IL-4+anti-IFN-γ), iTreg(TGF-β1+IL-2) and Th17(TGF-β1+IL-6+anti-IL-4+anti-IFN-γ) harvested from

day 3 cultures in the presence of appropriate cytokines along with αCD3(2μg/ml) and αCD28(1μg/ml).Densitometry was carried out for quantification of relative expression in various subsets using actin as a loading control. (B)The relative mRNA levels of *Prak* over the course of Th17 induction(n=4). (C) Western bot analysis of PRAK expression in CD19+ B and CD3+ T cells from the spleen (SP) or lymph nodes (LN) of WT and KO mice. Vinculin served as loading control. Quantification was performed using densitometry, setting PRAK level in WT cells as 1. (D to I) The frequency of IFNγ+(D and E), IL4+ and IL-5+ (F and G), and FOXP3+ (H and I) cells generated in day 3 cultures of WT or PRAK KO T cells (n=3) under Th1, Th2 and iTreg polarizing conditions, respectively (J and K). 7-AAD and Annexin V staining of Th17 cells generated in WT and KO cell cultures (n=3) in the presence of 5mM Z-VAD-FMK or the isovolumetric DMSO. (L) The frequency of IL-17A+ cells in Z-VAD-FMK treated WT and KO cell cultures(n=3). (M and N) Proliferation of WT and KO Th17 cells as measured by CFSE dilution (n=3). (O)Immunoblot of phosphorylated(S465/S467) and total SMAD2 in WT and KOTh17 cells (n=3). Quantification was performed using densitometry. (P)Heatmap for the expression of genes related to the JAK-STAT pathway in WT and KO Th17 cells. (Q)The relative mRNA levels of *RORγT* and *IL-23R* in WT and KO Th17 cells(n=4) as determined by quantitative RT-PCR. The statistics was performed using Student's t test. $*p < 0.05$; $***p < 0.001$; N.S. not significant.

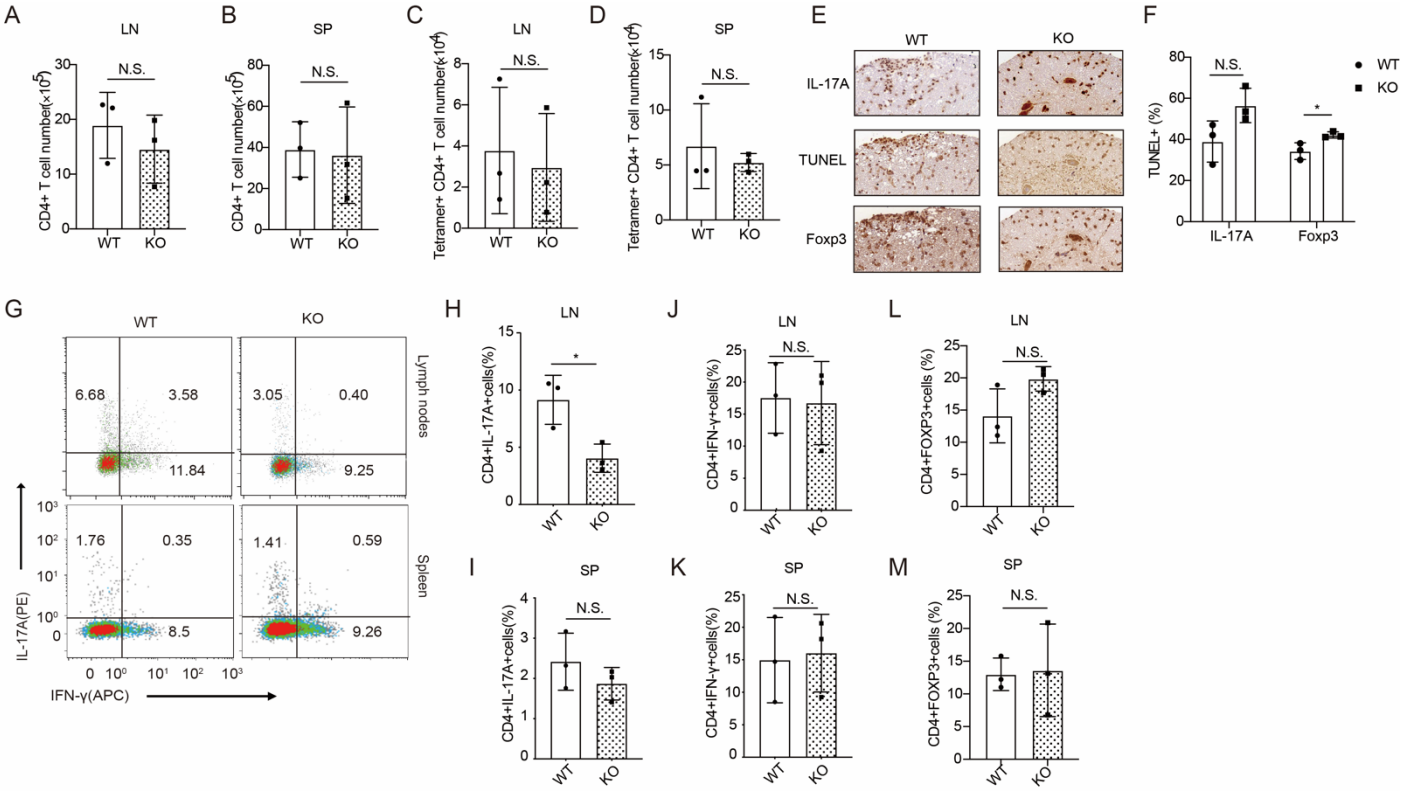


Fig. S2. Resistance to experimental autoimmune encephalomyelitis (EAE) in the absence of PRAK. (A-D) Absolute numbers of CD4+ T cells and tetramer+ CD4+ T cells in the lymph node and spleen of EAE mice. (E and F) Serial sections of the spinal cord from EAE mice were stained for TUNEL, IL-17A and Foxp3. Representative images are shown on the left. Statistical data of TUNEL+ cells among IL-17A+ and Foxp3+ cells are presented on the right. (G-K) Flow cytometric analyses were performed to detect IL-17 and IFN-γ production by CD4+ from the lymph node (n=3) and spleen (n=3) of EAE mice. Representative dot plots (G) are presented with statistical data of the percentage of CD4+IL-17+ (H and I) and CD4+IFN-γ+ (J and K) cells. (L and M). The percentage of CD4+Foxp3+ cells as detected by flow cytometry in the lymph node (n=3) (L) and spleen (n=3) (M). Statistics was performed using Student's t test. $*p < 0.05$; N.S. not significant.

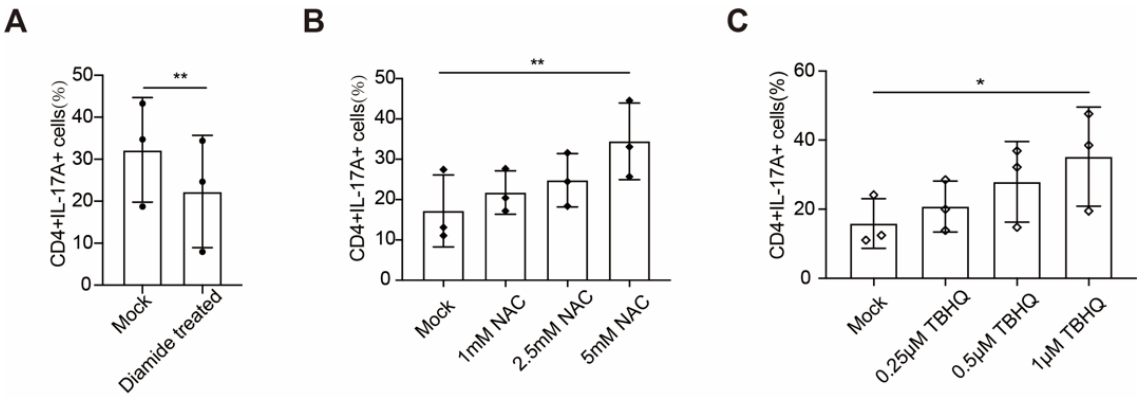


Figure S3. Inhibition of Th17 cell differentiation by ROS. (A) WT CD4+ T cells were briefly treated with 200μM diamide and then put into culture under Th17 polarizing conditions. Generation of IL-17+ cells was monitored by intracellular staining at day 3. (B and C) Generation of IL-17+ cells from WT CD4+ T cells in the presence of various concentrations of NAC (B) or tBHQ (C). Statistics was performed using paired Student's t test. $*p < 0.05$; $**p < 0.01$.

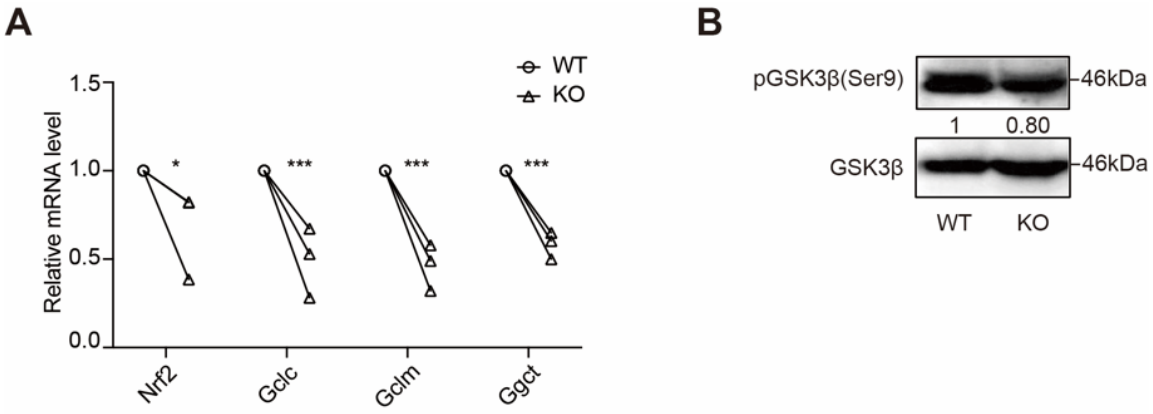


Figure S4. Reduced expression of anti-oxidant genes in *Prak*-deficient Th17 cells. (A)The relative mRNA levels of *Nrf2* and downstream *Gclc, Gclm* and *Ggct* in WT and KO Th17 cells were compared using quantitative RT-PCR (n=3). (B) Immunoblot of total and phosphorylated GSK3β (Ser9) of Th17 polarized for 3 days in vitro (n=3), Quantification was carried out using densitometry with total GSK3β serving as a loading control. Statistics was performed using Student's t test. $*p < 0.05$; $***p < 0.001$.

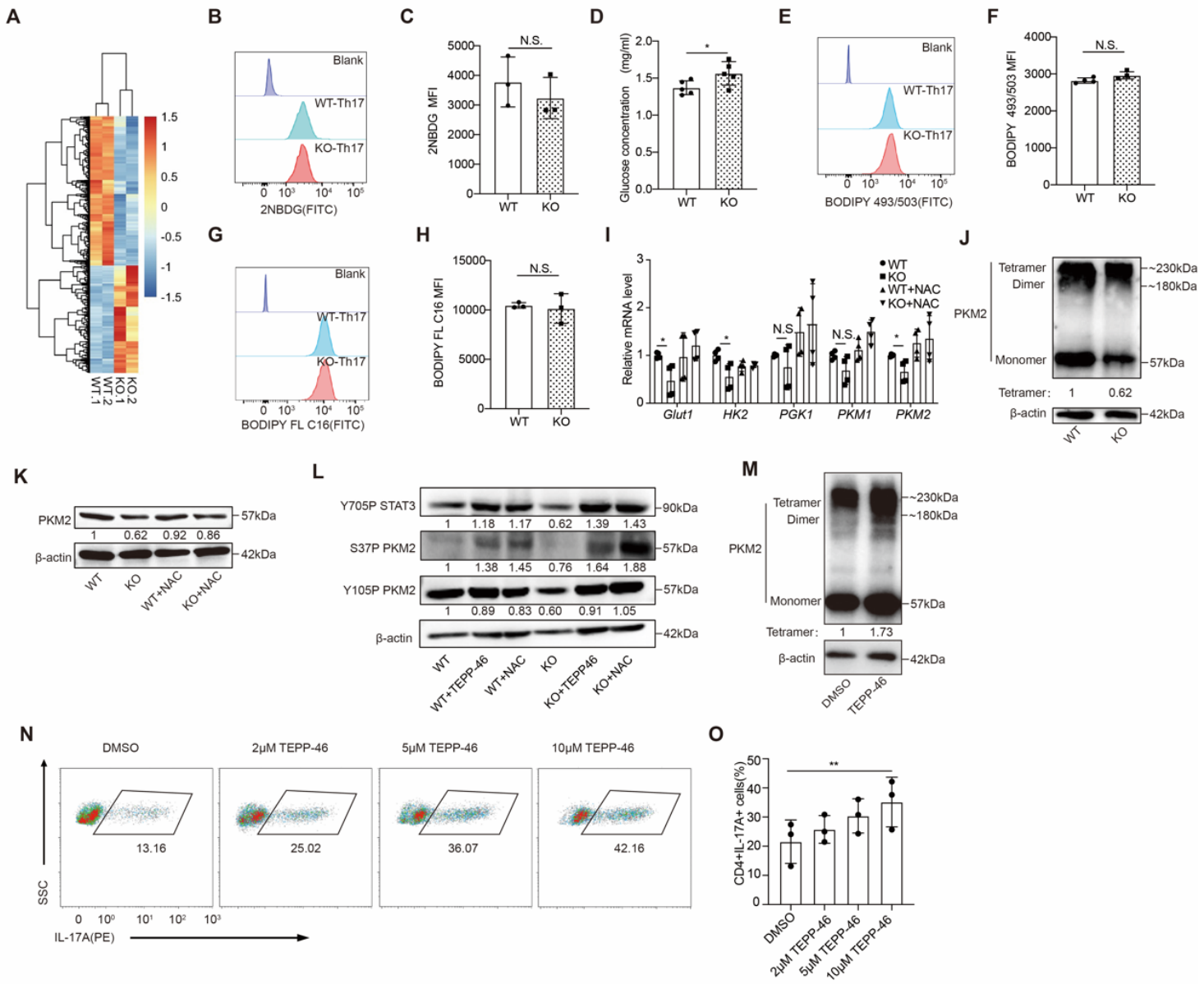
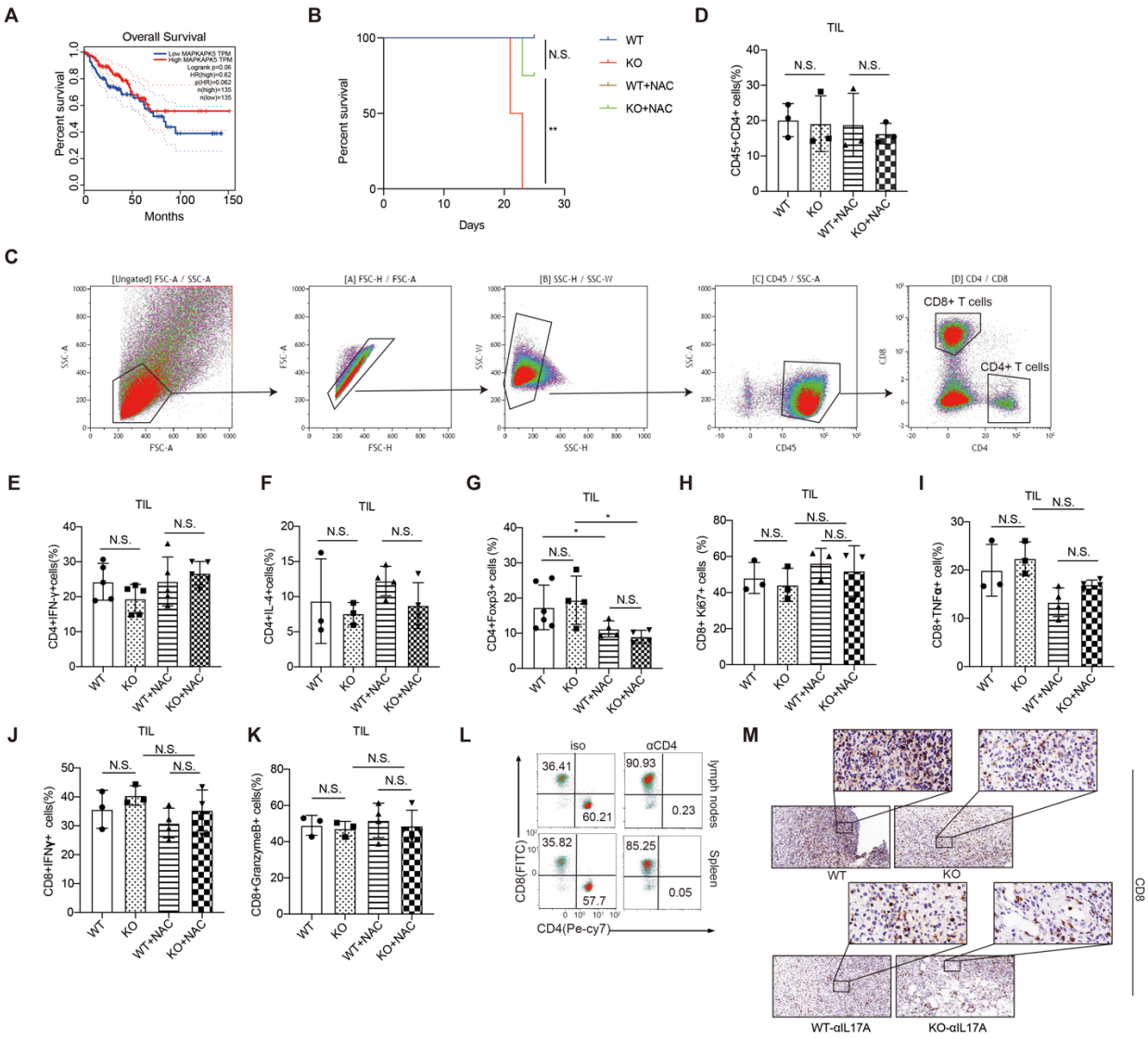


Figure S5. ROS accumulation in PRAK-deficient Th17 cells inhibits glycolysis and STAT3 phosphorylation in a PKM2-dependent way. (A)Heatmap of genes differentially expressed between WT and KO Th17 cells. (B and C) Intake of 2NBDG by WT and KO Th17 cells (n=3). (D) Glucose left in the supernatant of WT and KO Th17 cell cultures at day 3 of polarization (n=5). (E and F) The total contents of neutral lipids in WT and KO Th17 cells measured by BODIPY 493/503 (n=3). (G and H) Intake of BODYPY FL C16 by WT and KO Th17 cells (n=3). (I)The *GLUT1 HK2 PGK1 PKM1* and *PKM2* mRNA levels in WT and KO Th17 cells with or without treatment with 5mM NAC (n=3). (J) The tetramer, dimer and monomer isoform PKM2 in WT and KO Th17 cells following cross-link with 1mM Disuccinimidyl Suberate (DSS) (n=3). Quantification was performed using densitometry with β-actin as loading control. (K) Levels of total PKM2 in WT and KO Th17 cells in the presence or absence of NAC (n=3). Quantification was performed using densitometry with β-actin as loading control. (L) Phosphorylation of STAT3 and PKM2 in WT and KO Th17 cells in the presence or absence of NAC or TEPP-46 (n=3). Quantification was

performed using densitometry with β-actin as loading control. (M) The multiple isoforms of PKM2 in WT Th17 cells following treatment with TEPP-46 or isovolumetric DMSO (n=2). Quantification was performed using densitometry with β-actin as loading control. (N and O) TEPP-46 promotes Th17 polarization of *Prak*-deficient CD4+ T cells in a dose-dependent manner. Representative dot plots are shown in N. Data from 3 independent experiments are presented as Mean ± SD (O). The statistics was performed using Student's t test. $*p < 0.05$; $**p < 0.01$; N.S. not significant.



FigureS6. PRAK promotes anti-tumor immunity through regulating Th17 cell differentiation in colon cancer. (A)The survival curve of colon cancer patients grouped according to PRAK expression. Data were collected from Gene Expression Profiling Interactive Analysis (GEPIA). (B) The survival curve of tumor bearing WT and KO mice with or without NAC treatment (n=4 for each group). (C) Gating strategy for flow cytometric analysis of tumor infiltrating lymphocytes. (D-G) Frequencies of tumor infiltrating total CD4+ T cells (n=3) and CD4+IFN-γ+ cells(n=5), CD4+IL4+ cells(n=3/4) and CD4+Foxp3+ cells(n=5). (H-K) The percentage of Ki67+(H), TNFα+(I), IFNγ+(J), granzyme B+(K) cells among tumor-infiltrating CD8+ T cells(n=3). (L) Depletion of CD4+ T cells following treatment with anti-CD4. (M) IHC staining of CD8 in MC38 tumor sections from WT and KO mice with or without treatment with IL-17A neutralizing antibody. The statistics was performed using Student's t test. $*p < 0.05$; $**p < 0.01$; N.S. not significant.

SI References

1. X. Wang *et al.*, Transcription of Il17 and Il17f is controlled by conserved noncoding sequence 2. *Immunity* **36**, 23-31 (2012).
2. S. Angiari *et al.*, Pharmacological Activation of Pyruvate Kinase M2 Inhibits CD4(+) T Cell Pathogenicity and Suppresses Autoimmunity. *Cell Metab* **31**, 391-405 e398 (2020).
3. Z. Tang *et al.*, GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res* **45**, W98-w102 (2017).