

1 **Supplementary Information**

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3 **N-acetyltransferase 10 is implicated in the pathogenesis of cycling T cell-**
4 **mediated autoimmune and inflammatory disorders in mice**

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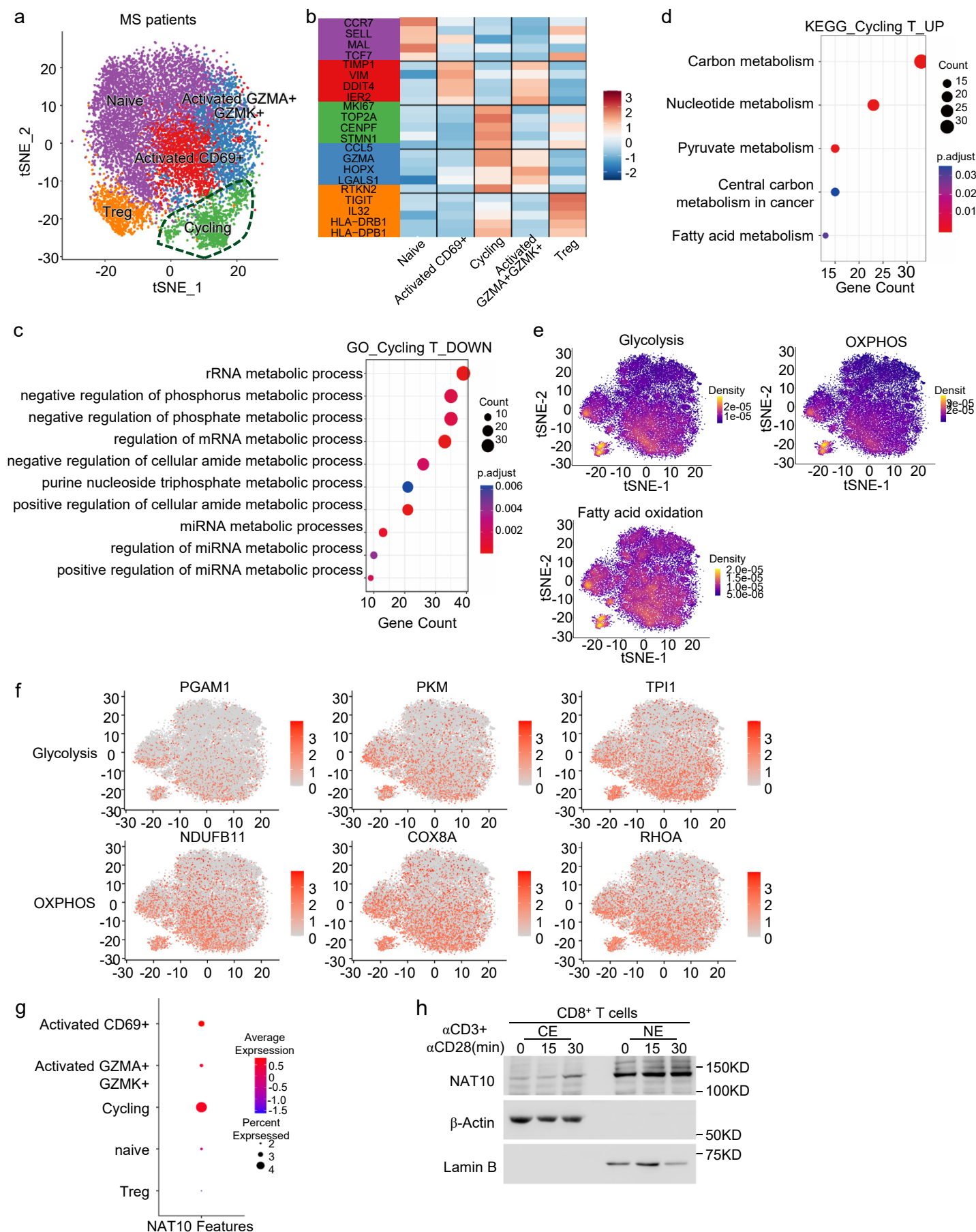
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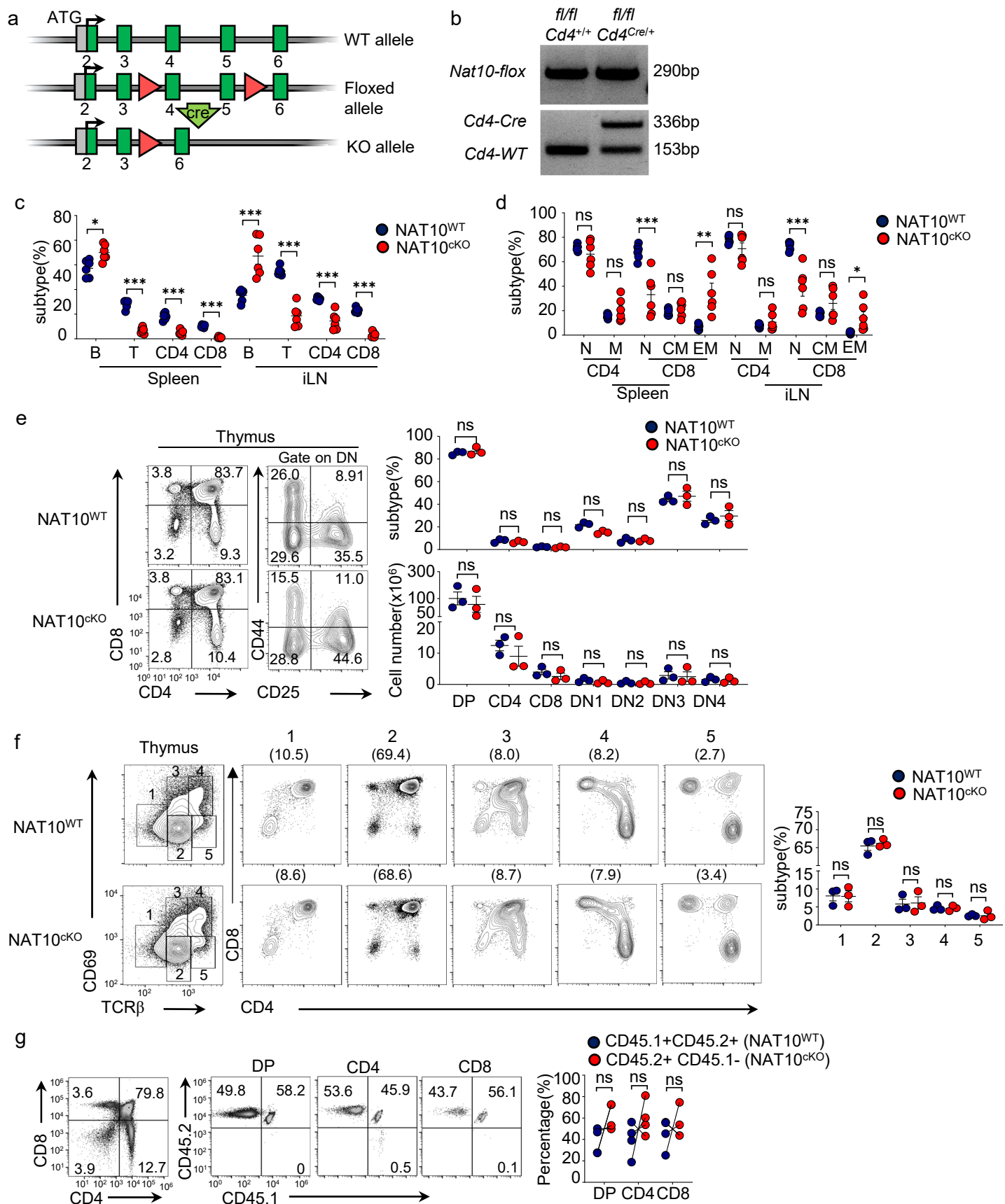
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21 This PDF file includes: **Supplementary Figures 1-9** and **Supplementary Tables 1**

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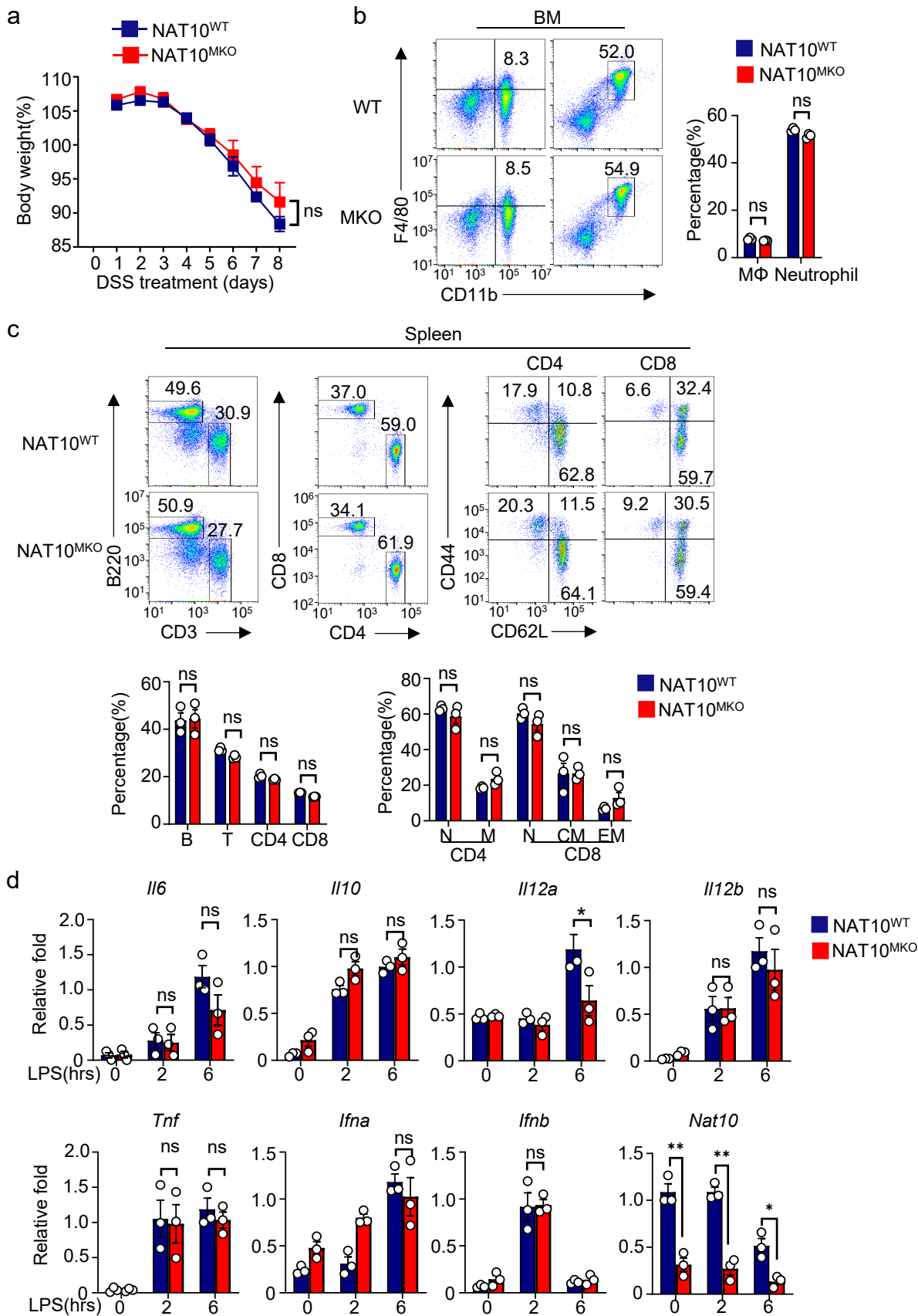
Supplementary Figure 1 The upregulation of NAT10 in cycling T cells involved in the onset of inflammatory diseases and T cell metabolism

(a) Single cell sequencing data of CD4⁺ T cell in cerebrospinal fluid of multiple sclerosis patient (n= 6) was required from Gene Expression Omnibus (GEO, accession code GSE138266), and clusters of immune cells were presented by t-distributed stochastic neighbor embedding (t-SNE) plot. (b) heatmap of the marker genes of clusters defined in (a). (c) Retrieved different expression genes (DEGs) between cycling and non-cycling T cells in CD samples were subjected to GO analysis, and the dot plot showing the enriched significant pathway. (d) The dot plot showing the enriched metabolic pathway subjected to GO analysis of Retrieved DEGs between cycling and non-cycling T cells. (e) irGSEA were used to visualize the characteristic different expression of metabolic gene set of indicated subgroups. (f) Metabolism-related gene expression projected on t-SNE plots. Red denotes high expression; grey denotes low expression. (g) Expression feature of NAT10 in different clusters of MS samples. (h) WT naïve CD8⁺ T cells were activated for indicated time by TCR crosslinking, the cytosolic and nuclear extracts were subjected to immunoblotting with antibody against NAT10.



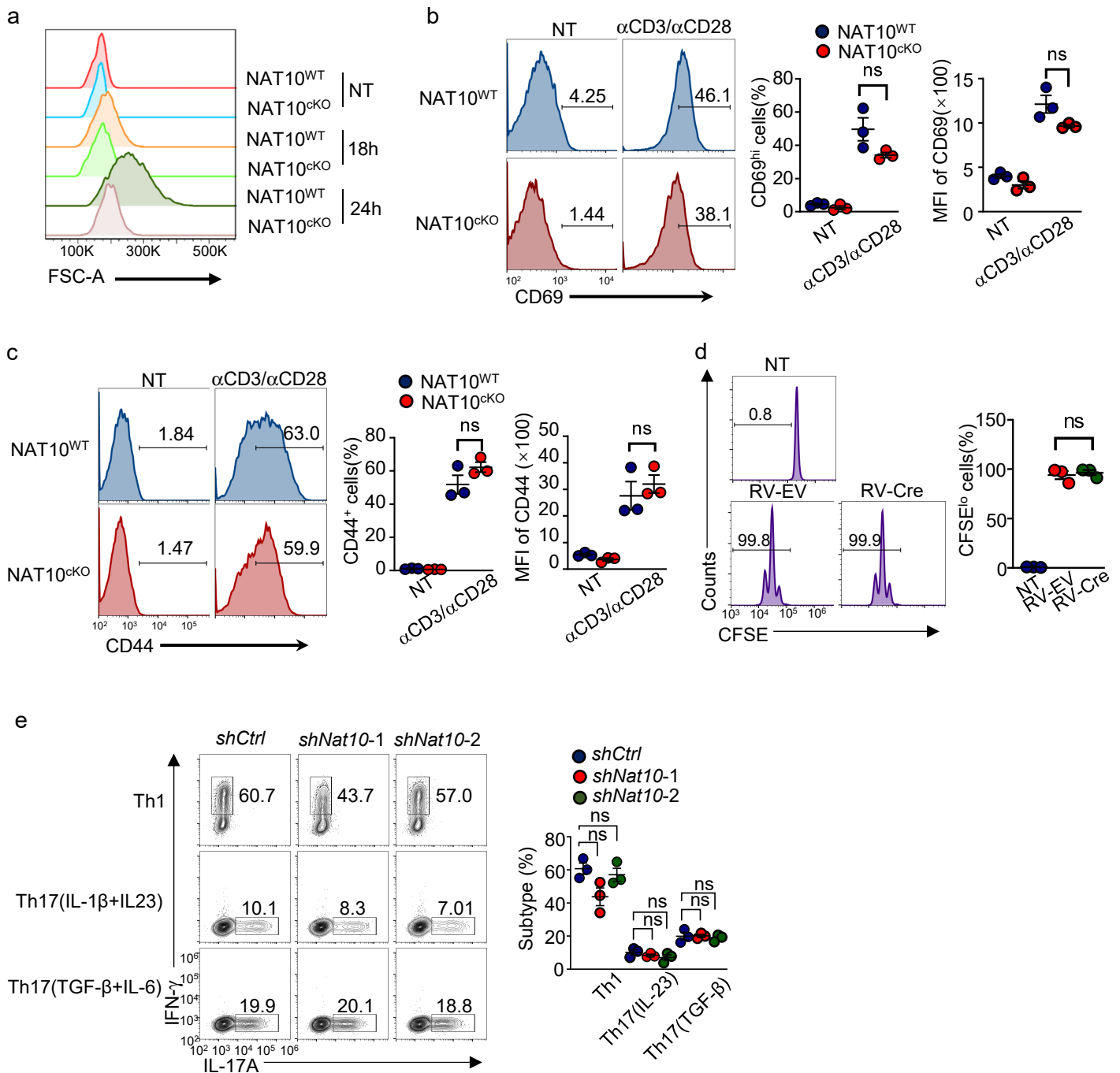
Supplementary Figure 2 NAT10 is critically involved in the maintenance of peripheral T lymphocyte

(a) Strategy of *Nat10*-floxed mice construction, and breeding strategy with *CD4^{Cre}* mice to generate NAT10^{cKO} mice. (b) Genotyping PCR of NAT10^{WT} (*Nat10^{fl/fl}*) and NAT10^{cKO} (*Nat10^{fl/fl}; Cd4-cre*) mice. (c) Percentage of indicated lymphocytes in inguinal lymph nodes (iLN) and spleen of NAT10^{WT} and NAT10^{cKO} mice. (d) Percentage of naïve and memory subtypes of CD4⁺ T cell and CD8⁺ T cell in iLN and spleen of NAT10^{WT} and NAT10^{cKO} mice. (e-f) FACS analysis of thymocyte subpopulations of NAT10^{WT} and NAT10^{cKO} mice. (g) Thymocyte subtype of bone marrow chimera mice were analyzed through FACS, and the results were represented as summary graph. Data were represented as mean ± S.D. Three independent experiments at least were performed in data represented above. Significance of the differences in c-g panels were identified by two-tailed unpaired t tests. *P < 0.05, **P < 0.01, ***P < 0.001, ns, no significant.



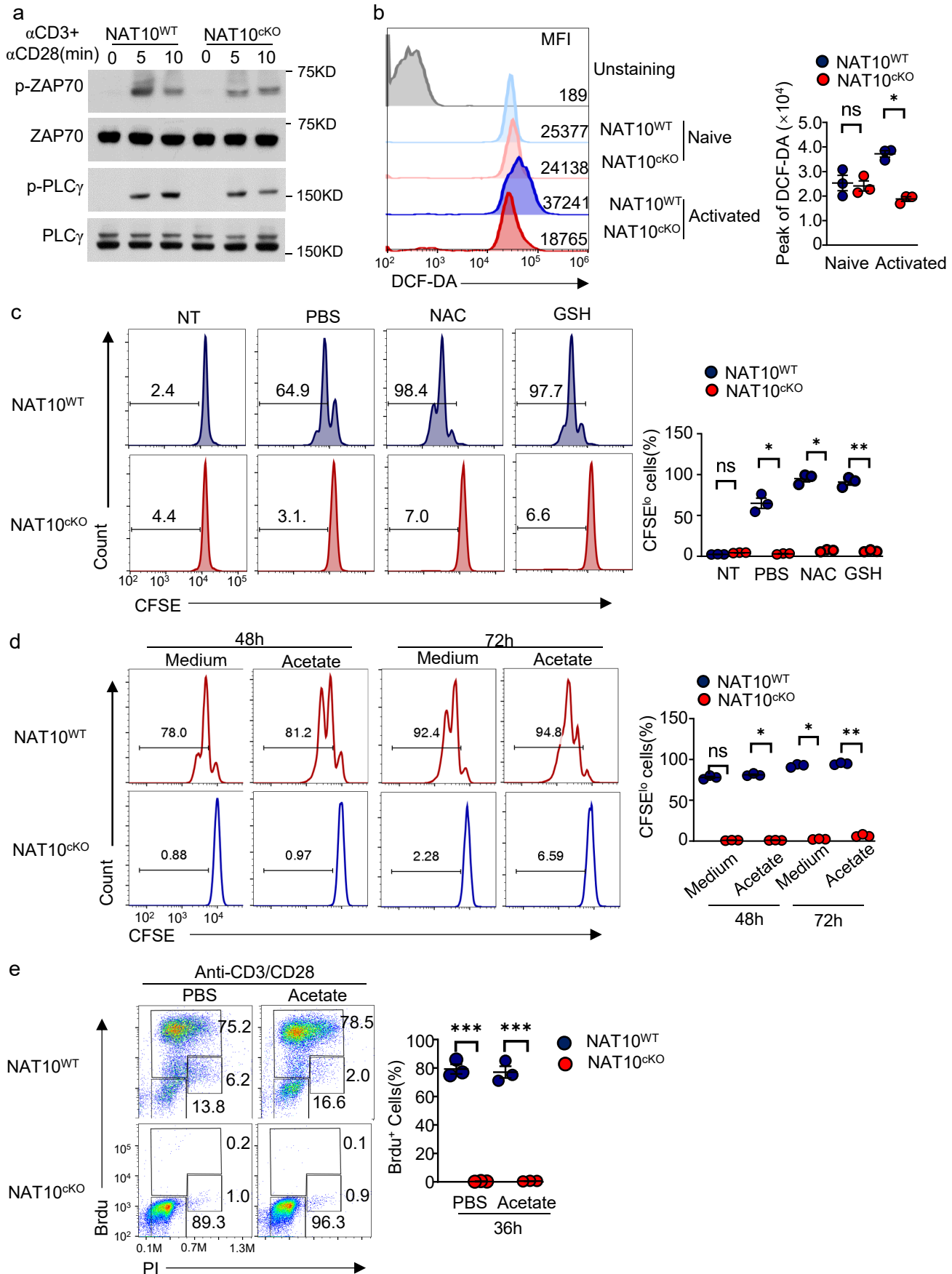
Supplementary Figure 3 NAT10 is not involved in the myeloid cells-driven colitis

(a) NAT10^{WT} and NAT10^{fllox/fllox}lyz2^{cre/+} (NAT10^{MKO}) mice were subjected to DSS-induced colitis for 5 days, and body weight were monitored until day 8. (b-c) FACS analysis of frequency of innate immunocytes (c) and lymphocytes (d) in the spleen of NAT10^{WT} and NAT10^{MKO} mice. (d) WT and NAT10-deficient bone marrow-derived macrophages (BMDM) were stimulated with 100 ng/mL LPS, subsequently the mRNA abundance of inflammatory cytokines and type I interferon coding genes were analyzed by RT-qPCR. These qPCR data are presented as fold change relative to the *Actb* mRNA level and normalized by Bio-Rad CFX Manager 3.1. Data were represented as mean $\pm$ S.D. Two independent experiments at least were performed in data represented above. Statistical significance was determined by multiple t-test (A) and one-way ANOVA with t test (b-d). ns, non-significant, *P < 0.05, **P < 0.01.



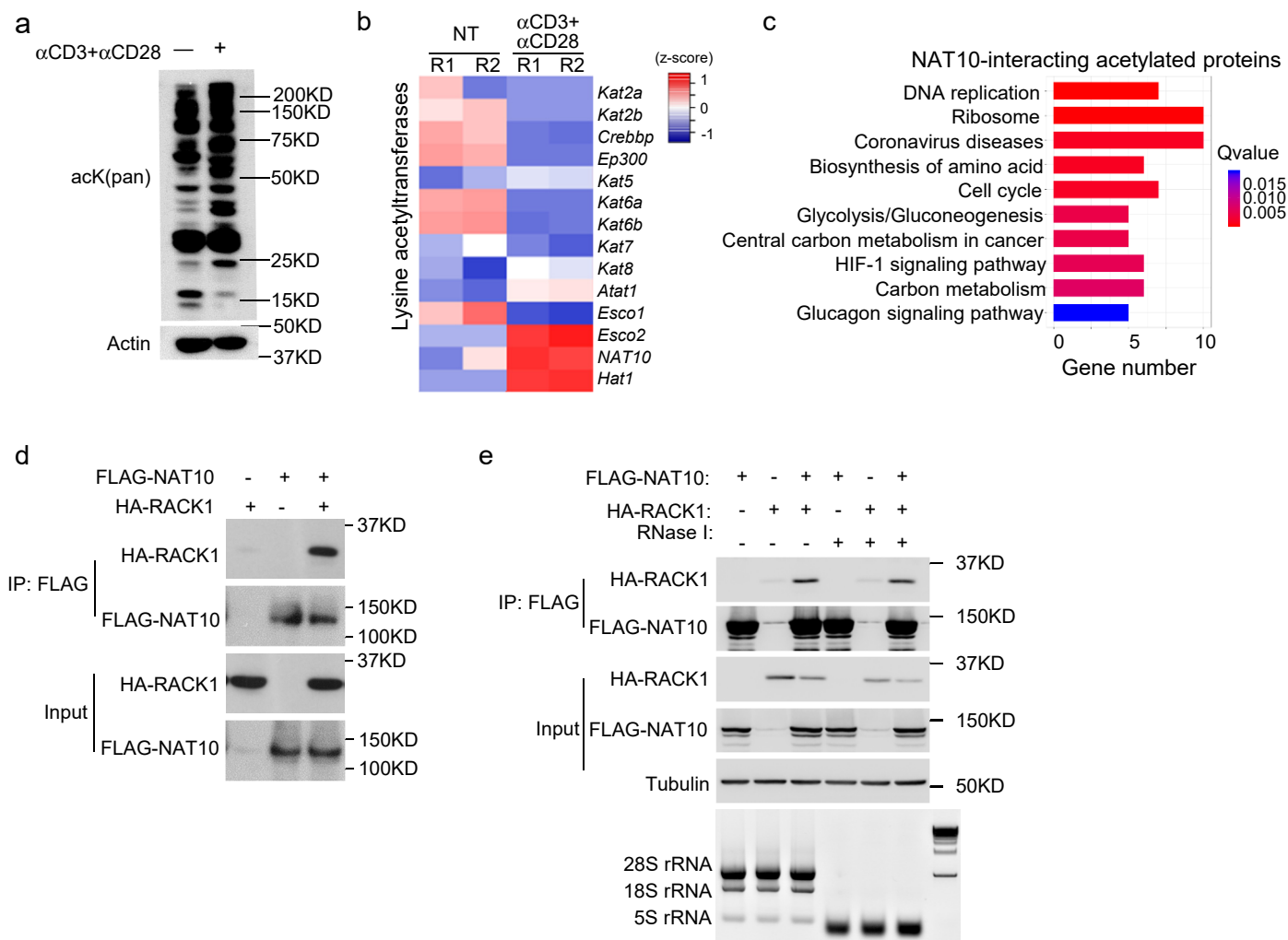
Supplementary Figure 4 NAT10 is dispensable at the late stages of T cell proliferation

(a) Forward side scatter (FSC) value of WT or NAT10-deficient naïve CD4⁺ T cells were recorded by FACS to measure the cell size. (b) FACS analysis of CD69 expression in WT or NAT10-deficient naïve CD4⁺ T cells stimulated with anti-CD3 plus anti-CD28 for 12h. (c) WT or NAT10-deficient naïve CD4⁺ T cells were stimulated with anti-CD3 plus anti-CD28 for 24h, and CD44 expression level was measured by FACS. (d) Naïve CD4⁺ T cells from NAT10 floxed mice were labeled with CFSE, activated for 24h and then infected with retrovirus carrying GFP or SV40NLS-Cre. The proliferation of these cells was measured with CFSE dilution by FACS after 48h. (e) Naïve CD4⁺ T cells were activated for 24h, and then infected with lentiviruses carrying NAT10-targeted shRNA or non-coding shRNA. These T cells were cultured in indicated polarization medium for additional 72h, the differentiation efficiency was analyzed by FACS. Data were represented as mean $\pm$ S.D. Three independent experiments at least were performed in data represented above. Statistical significance was determined by two-tailed unpaired t-test (b-c and e) and one-way ANOVA with t test (d). ns, no significant.



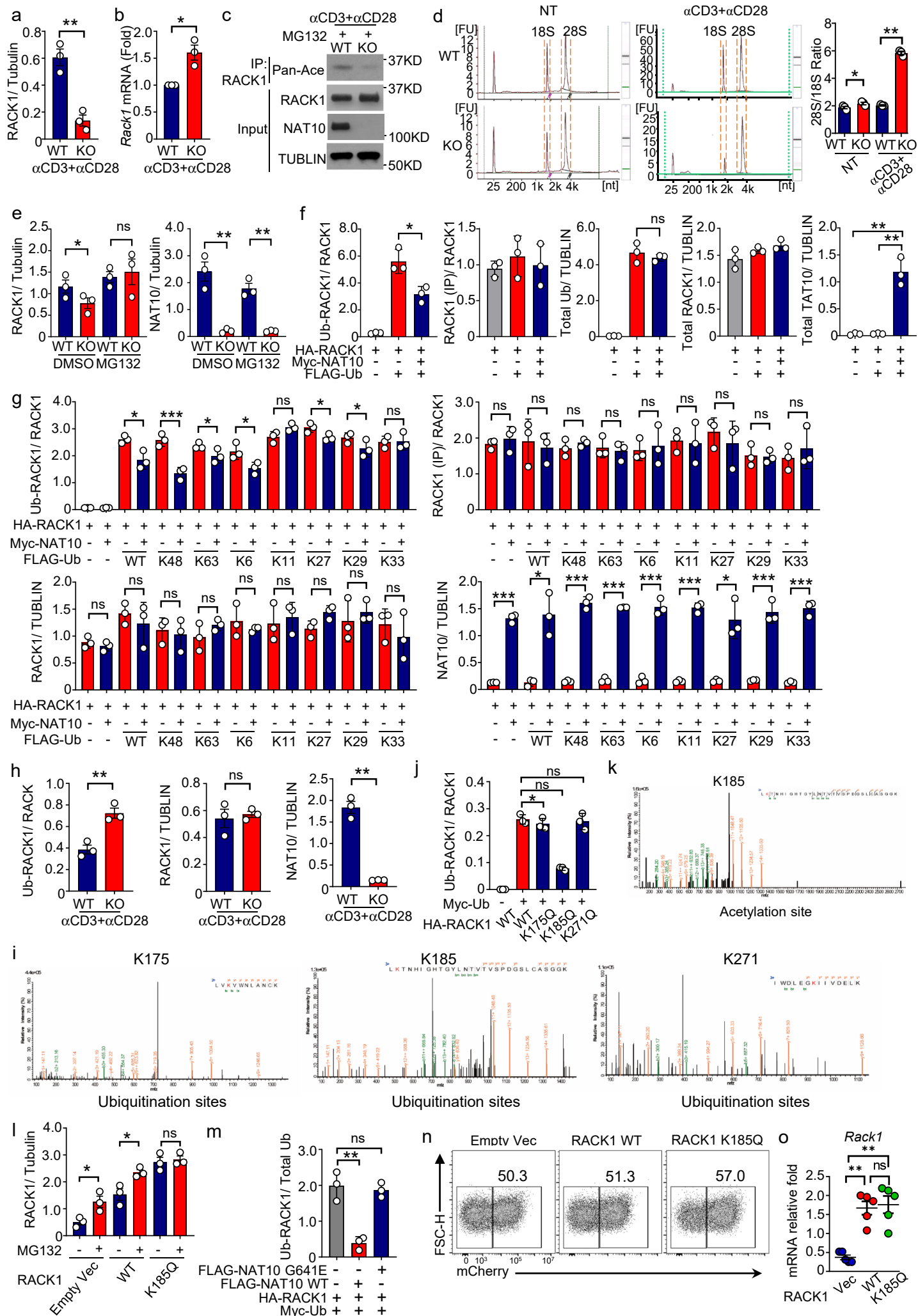
Supplementary Figure 5 NAT10 regulating the proliferation of T cell is independent of TCR proximal signaling and oxidative stress.

(a) Naive CD4⁺ T cells isolated from NAT10^{WT} and NAT10^{CKO} mice were stimulated with anti-CD3 plus anti-CD28 for indicated time. The phosphor-ZAP70 and phospho-PLC γ were analyzed by IB. (b) Naive or activated CD4⁺ T cells isolated from NAT10^{WT} and NAT10^{CKO} mice were incubated with 10 μ M DCFH-DA to analyzed the ROS production by FACS. (c) CFSE labeled WT or NAT10-deficient naive CD4⁺ T cells were cultured in complete medium containing ROS neutralizing reagents N-acetylcysteine (NAC) or glutathione (GSH). The proliferation of these cells was subsequently analyzed by CFSE dilution 72h later. (d-e) Naive CD4⁺ T cells isolated from NAT10^{WT} or NAT10^{CKO} mice were labeled with CFSE, and cultured in complete medium or complete medium containing 10 mM acetate. The proliferation (d) and cell cycle analysis (e) was measured at the indicated time points. Data were represented as mean $\pm$ S.D. Three independent experiments at least were performed in data represented above. Statistical significance was determined by two-tailed unpaired t-test (b-e). *P < 0.05, **P < 0.01, ***P < 0.005.



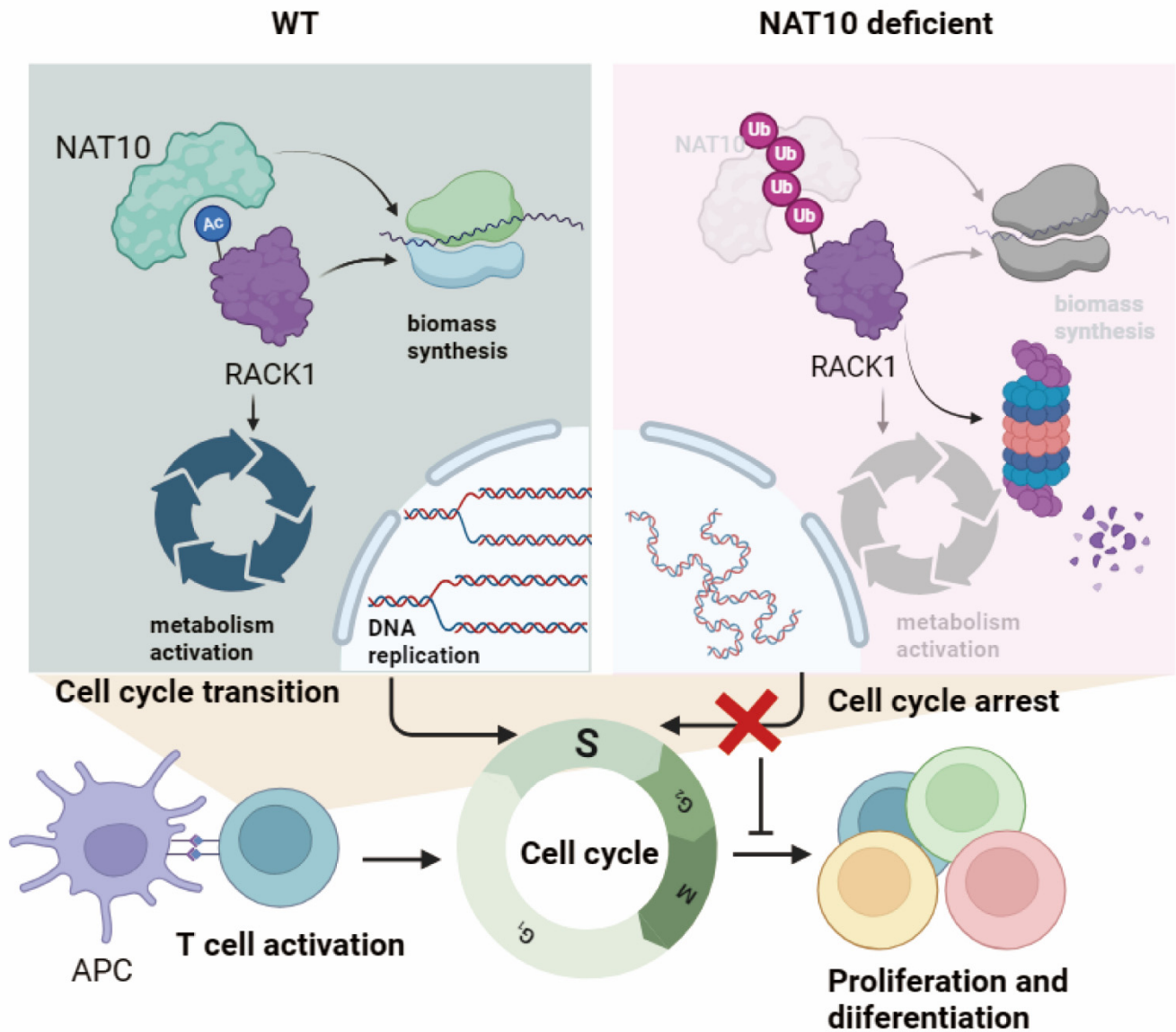
Supplementary Figure 6 NAT10 contributes to acetylation level of various proteins in activated T cells.

(a) Immunoblotting with pan-AcK antibody to analyze global protein acetylation level of WT naïve or activated CD4⁺ T cells. (b) Heatmap showing the classic lysine acetyl-transferases expression in WT CD4⁺ T cells with or without stimulation. (c) KEGG enrichment analysis showing significant enriched pathways of proteins interacted with NAT10. (d) RACK1 were isolated by immunoprecipitation (IP) and subjected to IB assays using FLAG (top panels) antibodies to evaluate the interaction between RACK1 and NAT10. Protein lysates were also subjected to direct IB (bottom panels). (e) HA-RACK1 and FLAG-NAT10 were co-expressed in HEK293T cells, followed by immunoprecipitation of HA-RACK1. The presence of immunoprecipitated NAT10 was analyzed using an anti-FLAG antibody. To assess the potential role of RNA in the interaction between RACK1 and NAT10, 20 units of RNase I were added to the cell lysate during immunoprecipitation. The efficiency of RNA digestion was confirmed by agarose gel electrophoresis (bottom panel). Data were represented as mean $\pm$ S.D. Three independent experiments at least were performed in data represented above. *P < 0.05, **P < 0.01, ns, no significant.



Supplementary Figure 7 NAT10 contributes to the acetylation of RACK1 protein.

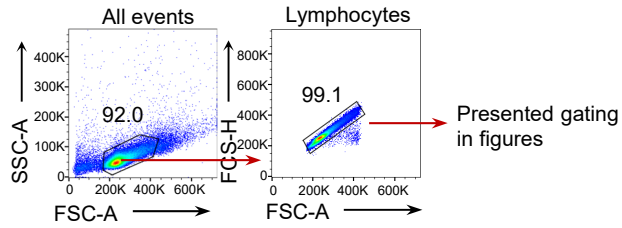
(a) The bar graph presented the semi-quantitative analysis results of the IB in the data of Fig. 7a. (b) The mRNA level of RACK1 in WT and NAT10-deficient activated CD4⁺ T cells were detected by RT-qPCR. These qPCR data are presented as fold change relative to the *Actb* mRNA level and normalized by Bio-Rad CFX Manager 3.1. (c) Total RNA was extracted from WT and NAT10-deficient CD4⁺ T cells, the abundance of 28S and 18S rRNA were measured by Agilent 2100 Bioanalyzer. (d-g) The bar graph presented the semi-quantitative analysis results of the IB in the data of Fig. 7b (d), Fig. 7c (e), Fig. 7d (f), and Fig. 7e (g), respectively. (h) K175, K185 and K271 ubiquitination of RACK1 in activated T cells identified by mass spectrometry. (i) The bar graph presented the semi-quantitative analysis results of the IB in the data of Figure 7H. (j) NAT10-mediated K185 acetylation of RACK1 *in vitro* was identified by mass spectrometry. (k-l) The bar graph presented the semi-quantitative analysis results of the IB in the data of Fig. 7k. The transfected efficiency of RACK1 WT and RACK1 mutant was detected based on mCherry by FACS. (m) The mRNA level of RACK1 in mCherry⁺ CD4⁺ T cells isolated as above were detected by RT-qPCR. These qPCR data are presented as fold change relative to the *Actb* mRNA level and normalized by Bio-Rad CFX Manager 3.1. (n) The bar graph presented the semi-quantitative analysis results of the IB in the data of Fig. 7m. Data were represented as mean $\pm$ S.D. Three independent experiments at least were performed in data represented above. Statistical significances were determined by two-tailed unpaired t-test (a, c-d, f and g), and others were determined by one-way ANOVA with multiple comparisons (e, i, k and m-n). *P < 0.05.



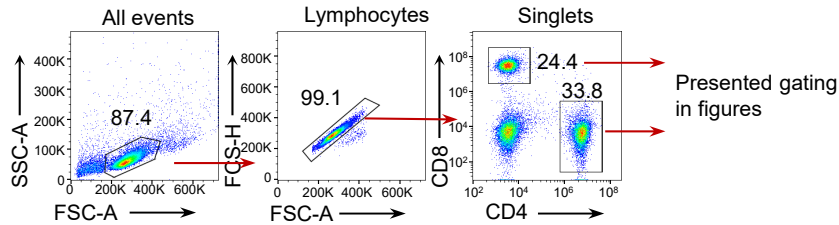
Supplementary Figure 8 Mechanistic diagram of the involvement of NAT10 in regulating T cell proliferation

In normal T cells, the catalytic activity of NAT10 plays a crucial role in the acetylation of RACK1, which stabilizes the RACK1 protein. This stabilization is essential for effective ribosome biogenesis and metabolic activation, enabling rapid cell proliferation and differentiation upon activation. In the absence of NAT10, RACK1 lacks the necessary acetylation modification, making it susceptible to ubiquitination and subsequent degradation via the proteasome pathway. Consequently, T cells fail to efficiently undergo ribosome biogenesis and metabolic activation, leading to significantly impaired proliferation and differentiation.

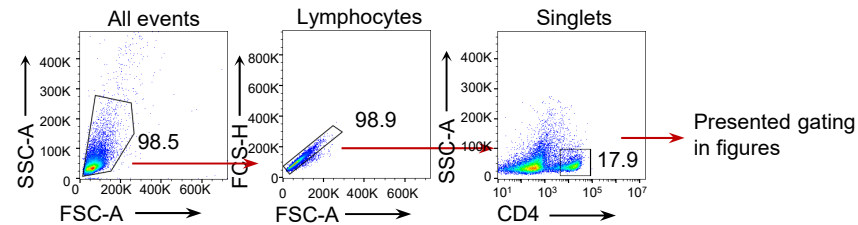
- a Gating strategy for Fig 2a, 2e, 3A-B, 3F, 6a-b, 7n, 7r, S2e-g, S3b-c, S4a-e, S5b-e, S7l



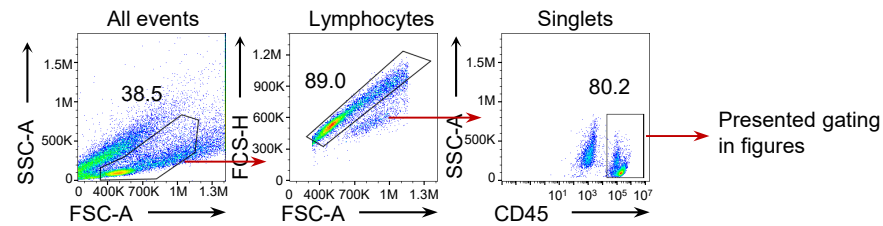
- b Gating strategy for Fig 2b,2d



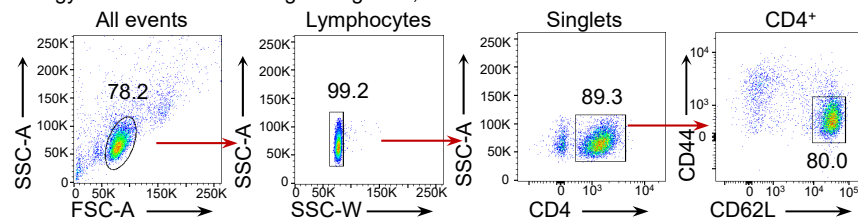
- c Gating strategy for Fig 2c



- d Gating strategy for Fig 3d-e, 3g



- e Gating strategy for Naive T cell sorting for Fig 4a-d, 4f



Supplementary Figure 9 FACS sequential gating/sorting strategies

(a-e) The gating/sorting strategies for indicated FACS data of indicated Figure in different panels.

RT-qPCR primers		
Gene	Forward primer	Reverse primer
<i>Ccne1</i>	GTGGCTCCGACCTTTCAGTC	CACAGTCTTGTCATCTTGGCA
<i>Cdk4</i>	CATACCTGGACAAAGCACCTCC	GAATGTTCTCTGGCTTCAGGTCC
<i>Cdkn1a</i>	CCTGGTGATGTCCGACCTG	CCATGAGCGCATCGCAATC
<i>Tp53</i>	CAGTCTGGGACAGCCAAGTC	TGACCCACAACCTGCACAGG
<i>Rack1</i>	CCATGAGCGCATCGCAATC	CACGCTCAACACATCCTTGGTG
<i>Actb</i>	CGTGAAAAGATGACCCAGATCA	CACAGCCTGGATGGCTACGT
<i>Il6</i>	CACAGAGGATACCACTCCCAACA	TCCACGATTTCCAGAGAACA
<i>Il10</i>	CCAGAGCCACATGCTCCTAGA	GGTCCTTTGTTTGAAAGAAAGTCTTC
<i>Il12a</i>	ACTAGAGAGACTTCTTCCACAACAAGAG	GCACAGGGTCATCATCAAAGAC
<i>Il12b</i>	GGAGACACCAGCAAAACGAT	TCCAGATTACAGACTCCAGGG
<i>Tnf</i>	CATCTTCTCAAAATTCGAGTGACAA	CCAGCTGCTCCTCCACTTG
<i>Ifna</i>	AAGTATTTCTCACAGCCAGCAG	AGTCCATCAGCAGCTCAATGAC
<i>Ifnb</i>	AGCTCCAAGAAAGGACGAACAT	GCCCTGTAGGTGAGGTTGATCT
<i>Nat10</i>	CACAAACATTGCTACTGCTACT	AACGCTTCAAAATCCTGGAGG

Genotyping primers		
Target gene	Forward primer	Reverse primer
<i>Nat10</i> flox genotyping	GGAACCATGAGTATTGTAGCCTGC	CTATTGGCTGTGACTTCAGCAGAC
<i>Cd4</i> -Cre genotyping WT	GTTCTTTGTATATATTGAATGTTAGCC	TATGCTCTAAGGACAAGAATTGACA
<i>Cd4</i> -Cre genotyping mutant	GTTCTTTGTATATATTGAATGTTAGCC	CTTTGCAGAGGGCTAACAGC
<i>Lyz2</i> cre genotyping WT	CTTGGGCTGCCAGAATTTCTC	TTACAGTCGGCCAGGCTGAC
<i>Lyz2</i> cre genotyping mutant	CTTGGGCTGCCAGAATTTCTC	CCCAGAAATGCCAGATTACG

Supplementary Table 1 Oligonucleotides used for RT-qPCR and genotyping. cDNA prepared from RNA of CD4⁺ naïve and activated T cells was used to detect target genes by RT-qPCR with indicated primers. Genotype of mice were determined by standard PCR using specific primers listed in the table.