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Supplementary Information for

Transcription factor EB coordinates environmental cues to regulate T regulatory cells mitochondrial fitness and function

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Supplementary material and method
Figures S1 to S7

Supplementary material and method

Mice

Foxp3$^{YFP\text{-}Cre}$ transgenic mice, *Cd4*^{Cre} transgenic mice and *Rag2*$^{-/-}$ mice were obtained from The Jackson Laboratory. *Tcfeb*$^{fl/fl}$ mice were generated by CRISPR/Cas9 technology. In brief, two *loxp* sites were inserted into upstream of exon 5 and downstream of exon 7 of *Tcfeb*. sgRNAs to target either a region upstream of the exon 5 (sgRNA: 5'-TTTGTACAAGTCCTAGGGAGGGG-3') or downstream of the exon 7 (sgRNA: 5'-TGTCTTGACGCAGTAGGAGCAGG-3') were cloned into the pCS plasmid and then were assessed for on-target activity using a Universal CRISPR Activity Assay (UCATM, Biocytogen Pharmaceuticals). Then, *Cas9* mRNA, targeting vector, and sgRNAs were co-injected into the cytoplasm of one-cell stage fertilized C57BL/6 eggs. The injected zygotes were transferred into oviducts of Kunming pseudopregnant females to generate F0 mice. The *Tcfeb*$^{fl/fl}$ mice were crossed with *Cd4*^{Cre} transgenic mice or *Foxp3*$^{YFP\text{-}Cre}$ transgenic mice in C57BL/6 background to produce age-matched *Cd4*^{Cre}*Tcfeb*$^{fl/fl}$ mice, *Foxp3*$^{YFP\text{-}Cre}$*Tcfeb*$^{fl/fl}$ mice, and control mice for experiments. Mice were maintained in a specific pathogen-free facility, and all animal experiments in accordance with guidelines were approved by the Institutional Animal Care and Use Committee of Tongji Medical College, HUST.

T cell-transfer colitis

$CD4^+CD25^+YFP^+$ Treg cells were sorted from the spleens and lymph nodes of *Foxp3*$^{YFP\text{-}cre}$ and *Foxp3*$^{YFP\text{-}cre}$*Tcfeb*$^{fl/fl}$ mice (6-8-wk-old) and $CD45RB^{hi}$ $CD25^-CD4^+$ T cells were sorted from the spleens and mesenteric lymph nodes of WT congenic mice. 2×10^5 naïve $CD4^+$ T cells mixed with 2×10^5 WT or *Tcfeb*-deficient Treg cells were transferred *i.p*. into *Rag2*$^{-/-}$ mice. Recipient mice were weighted weekly and analyzed 12 weeks after transfer.

BM chimera

Bone marrows were isolated from *Foxp3*$^{YFP\text{-}cre}$ ($CD45.1^+CD45.2^+$) mice or age-matched *Foxp3*$^{YFP\text{-}cre}$*Tcfeb*$^{fl/fl}$ ($CD45.1^-CD45.2^+$) mice. $CD45.1^+$ mice (10-wk-old) were sub-lethally irradiated with 5 Gy and intravenously injected with a 1:1 mixture of BM cells (5×10^6 cells per mouse). Mice were analyzed at 8 weeks after reconstitution.

RNA-sequencing analysis

Wild-type or *Tcfeb*-deficient naive $CD4^+$ T cells were isolated from age-matched *Tcfeb*$^{fl/fl}$ and $CD4^{Cre}$ *Tcfeb*$^{fl/fl}$ mice using negative magnetic selection (Mouse naïve $CD4^+$ T Cell Isolation Kit; Miltenyi). Naive $CD4^+$ T cells were differentiated under Treg conditions for 60 h. 2×10^6 Treg cells were used for total RNA isolation with TRIzol (Invitrogen) and subjected to RNA-sequencing using Illumina Nextseq 500. The raw reads were aligned to the mouse reference genome (version mm10), using Tophat2 RNASeq alignment software. Differentially expressed transcripts were identified by limma (1) based on moderated *t*-test,

and the Benjamini-Hochberg method was used to adjust the *p* value. R package clusterProfiler (2) was used to identify enriched pathways in gene ontology (GO) analysis. Lists of differentially expressed genes with a cut-off of *p* value < 0.05 were selected to identify enriched biological processes. The raw RNA-seq data were deposited in the Genome Sequence Archive at National Genomics Data Center under accession number CRA007442 that are publicly accessible at https://ngdc.cn.cb.ac.cn/gsa.

Metabolic analysis

Seahorse XFe96 Extracellular Flux Analyzer (Agilent Technologies) was used for metabolic experiments. WT or *Tcfeb*-deficient naive $CD4^+$ T cells were incubated with plate-bound anti-CD3 plus anti-CD28 (5 μg/ml of each), anti-IL-4 and anti-IFN-γ (10 mg/ml of each), TGF-β (5 ng/ml) and hIL-2 (10 ng/ml) for 72 h. Cells were washed and resuspended with Seahorse XF Base Medium (Agilent Technologies) supplemented with 2 mM L-glutamine and 1 mM sodium pyruvate and 1×10^6 cells were seeded into one well of the 96-well seahorse assay plate 12 h before seahorse testing. The ECAR and OCR for each well were determined. Intracellular amounts of ATP in Treg cells differentiated from WT or *Tcfeb*-deficient naive $CD4^+$ T cells were measured using luminescent ATP detection assay kit (Beyotime Biotechnology) according to the manufacturer's instructions.

T cell differentiation and retroviral transduction

Naive $CD4^+CD44^{low}CD62L^{high}$ T lymphocytes were sorted with FACS Aria II after enrichment with $CD4^+$ T cell negative isolation kit (Miltenyi Biotec). T cells were cultured with RPMI 1640 complete medium containing supplemented with 10% (vol/vol) heat-inactivated FBS (Gibco), 1% (vol/vol) penicillin-streptomycin, 1 mM sodium pyruvate (Thermo Fisher Scientific), 1×NEAA (Thermo Fisher Scientific) and 50 μM β-mercaptoethanol (Millipore Sigma). For Th0 conditions, naive $CD4^+$ cells were stimulated with plate-bound anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) in the presence of anti-IL-4 (10 μg/ml) and anti-IFN-γ (10 μg/ml). For Th17 conditions, TGF-β (2 ng/ml), IL-6 (10 ng/ml), anti-IL-2 (10 μg/ml), anti-IL-4 (10 μg/ml) and anti-IFN-γ (10 μg/ml) were included in cultures. For Treg cell differentiation, TGF-β (5 ng/ml), hIL-2 (10 ng/ml), anti-IL-4 (10 μg/ml) and anti-IFN-γ (10 μg/ml) were added for 3 days.

TFEB-S211A retrovirus was described previously (3). Full-length cDNA encoding Myc and TFEB were PCR-amplified, TFEB was then tagged with HA by nest PCR amplification. Myc and HA-TFEB was cloned in to MSCV-IRES-EGFP (MIG) vector by enzyme digestion, followed by or homologous recombination. For generation of retroviral particles, HEK293T cells were grown to a confluency of 80-90% and were transfected with retroviral constructs and pCL-Eco plasmid at a 2:1 ratio using lipofectamine 2000. Viruses were collected at day 2-3 after transfection. For retrovirus transduction, naive $CD4^+CD44^{low}CD62L^{high}$ T lymphocytes were activated by plate-bound anti-CD3 (5 μg/ml,

Bio X Cell) plus anti-CD28 (5 μg/ml, Bio X Cell) in 48-well plates for 24 h and then transduced with the retroviral particles in the presence of 12 μg/ml polybrene by centrifugation at 2000 rpm for 2 h. After transfection, T cells were cultured under Treg differentiation conditions as described above.

Mitochondrial morphology and membrane potential analysis

For electron microscopy analysis of mitochondrial morphology, 5×10^6 Treg cells were fixed in 2.5% glutaraldehyde (Sigma Aldrich) in 0.1 M phosphate buffer (pH 7.4, pre-warmed at 37°C for 5 min) at room temperature followed by 2 h on ice. After fixation, cells were rinsed 5 times for 1 min each in 0.1 M phosphate buffer, then post-fixed in 2% OsO4 in 0.1 M phosphate buffer for 2 h. After 3 times wash for 15 min each in 0.1 M phosphate buffer (pH 7.4), cell were dehydrated at room temperature with graded ethanol series as followed (30%, 50%, 70%, 80%, 90%, 100%) for 15 min each. Samples then undergo resin penetration and embedding with EMBed 812 (SPI). After polymerization in a 65°C oven for 48 h, sections were cut to 60-80 nm thin slide on the ultramicrotome (Leica UC7), and fished out onto the 150 meshes cuprum grids. The cuprum grids were then stained with 2% uranium acetate saturated alcohol solution and 2.6% Lead citrate in turn. After staining, the cuprum grids are observed under HT-7700 electron microscope (Hitachi HT-7700) at an accelerating voltage of 80 KV, Images were taken at either 2500× or 7000× magnification.

For analysis of mitochondrial membrane potential, MitoTracker Deep Red FM (Thermo Fisher Scientific), MitoTracker Green FM (Thermo Fisher Scientific) and TMRE (tetramethylrodamine, ethyl ester, Thermo Fisher Scientific) were used. MitoTracker Deep Red FM and TMRE were both membrane potential dependent mitochondrial dyes. In brief, 1×10^6 Treg cells washed twice by ice-cold PBS, were incubated with MitoTracker Deep FM (50 nM), MitoTracker Green FM (50 nM) or TMRE (20 nM) for 20 min in PBS at 37 ℃. Cells were washed and the indicated dyes were detected by flow cytometry.

Real-time PCR analysis

Cells were harvested and total RNAs were extracted using TRIzol (Invitrogen), cDNA was synthesized with cDNA Reverse Transcription Kit (TOYOBO) and analyzed using a Bio-Rad SYBR Green intercalating fluorophore system (Applied Biosystems). Data were normalized to the expression of β-actin using $^{\Delta\Delta}$CT.

Flow cytometry

Cells were stained in PBS containing 0.5% FBS with indicated fluorochrome-conjugated antibodies for surface marker analysis. For intracellular cytokine expression analysis, cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Millipore Sigma), ionomycin (500 ng/ml, Millipore Sigma), together with Golgi-plug (BD Bioscience) for 4

h and stained with a Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Bioscience) according to the manufacturer's recommendations. Intranuclear protein (Foxp3, Ki67) staining was performed with Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) according to the manufacturer's instructions after cell surface staining. Phospho-protein staining of p-S6 was performed according to the manufacturer's recommendations.

Immunoblot analysis

Cells were lysed using RIPA lysis buffers containing protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Millipore Sigma), for 30 min on ice and then centrifuged at 12000 g for 10 min at 4 °C. Then protein concentrations in samples were quantified by BCA assay before loading the samples for electrophoresis and membrane transfer. The blots were probed with the following primary antibodies overnight at 4 °C: anti-TFEB (Abcam), anti-p-$S6^{Ser235/Ser236}$ (D57.2.2E, CST), anti-p-$STAT5^{Tyr694}$ (C71E5, CST), anti-S6 (54D2, CST), anti-STAT5 (D2O6Y, CST), anti-STAT5 (D2O6Y, CST), anti-LC3A/B (D3U4C, CST), anti-Cathepsin B (D1C7Y, CST), anti-p-$TFEB^{Ser211}$ (E9S8N, CST). All blots were then incubated with corresponding secondary antibodies for subsequent enhanced chemiluminescent (ECL) exposure (RPN2232, GE healthcare). The band intensities of all the immunoblot were analyzed by ImageJ software.

Immunofluorescence

For measuring TFEB expression upon TCR and IL-2 stimulation, purified naïve $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) for 24 h. For detection of LC3 puncta, naïve $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) for 60 h under Treg differentiation condition. Afterwards, cells were fixed with 4% paraformaldehyde for 10 min. Fixed samples were permeabilized for 20 min by 0.5% Triton-X. After blocking with 5% BSA in PBS for 1 h. Primary antibodies were used as followed: anti-TFEB (D4L2P, CST), anti-LC3A/B (D3U4C, CST), anti-CD4 monoclonal antibody (RM4-5, Thermo Fisher Scientific).

Histology

After induction of colitis, small intestines were collected and fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned for staining with hematoxylin and eosin.

Chromatin-immunoprecipitation assay

For H3K4me3 immunoprecipitation, in vitro differentiated Treg cell were used. For HA-tag immunoprecipitation, retroviral transduction was performed before T cells were cultured under Treg cell differentiation condition. Cells were harvested and treated with 1% formaldehyde for 15 min for fix and crosslinking. The crosslinking reactions was stopped by adding glycine to a final concentration of 125 mM and incubating for 5 min at room temperature. Afterwards, 6×10^6 cells were lysed and Chromatin was fragmented by

sonication. Chromatin was then immunoprecipitated with 2 μg of anti-HA (CST, C29F4), anti-H3K4me3 (Abcam, Ab8580) or IgG control antibodies at 4 °C overnight. After washing and elution, crosslinks were reversed for 4 h at 65°C. The eluted DNA was purified and analyzed by qPCR using a Bio-Rad SYBR Green intercalating fluorophore system.

Primers used to amplify the H3K4me3 enriched region of foxp3 promoter were F, 5′-AACACAATGCTGTCTCTACCTGCC-3′, R, 5′-CCCTCACCACAGAGGTAAAAGG-TA-3′. Primer used to amplify TFEB binding site in Myc promoter were F, 5′-GCAAA-TCCCGAGGGAATATGCAT-3′, R, 5′ CCTGAATACTACGCTGTGCATTCT-3′.

Mice starvation

C57/BL6 mice were starved for 24 h, TFEB expression in spleens was determined by immunofluorescent staining. For the immunoblot assay of TFEB expression in $CD4^+$ T cells, mice were starved for 36 h and $CD4^+$ T lymphocytes were isolated from spleens using $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec). For the detection of Treg in periphery blood, C57/BL6 mice were starved for 48 h.

***In vitro* Treg suppression assay**

$CD4^+CD25^+YFP^+$ Treg cells were sorted from spleens of *Foxp3*$^{YFP\text{-}cre}$ or *Foxp3*$^{YFP\text{-}cre}$*Tcfeb*$^{fl/fl}$ mice with FACS Aria II. Sorted naive $CD4^+$ T cells were labeled with CellTrace Violet (CTV, Thermo Fisher Scientific). CTV-labeled naïve cells ($1x10^5$ cells per well) were cultured alone or at different ratios of sorted wild-type or *Tcfeb*-deficient Treg cells in the presence of plate-coated anti-CD3 (1μg/ml) and anti-CD28 (1 μg/ml) antibodies. On day 3, cells were harvested and analyzed by flow cytometry.

For $CD8^+$ T cells proliferation suppression assay, $CD8^+$ T cells were isolated from WT mice using negative magnetic selection (Mouse $CD8^+$ T Cell Isolation Kit; Miltenyi) and stained with CellTrace CFSE (Thermo Fisher Scientific). CFSE-stained $CD8^+$ T cells (1×10^5 cells/well) were cultured alone or together with FACS-sorted wild-type or *Tcfeb*-deficient $CD4^+CD25^+YFP^+$ Treg cells in the presence of plate-coated anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) antibodies. On day 3, cells were harvested and analyzed by flow cytometry.

Tumor models and isolation of TILs

Isolated tumor-derived cell lines (TDCLs) lung cancer cell line was provided by Dr. Shuguo Sun (4). In brief, TDCLs derived from a *Kras*$^{LSL\text{-}G12D/+}$; *Tp53*$^{fl/fl}$ conditional NSCLC model, were cultured in RPMI 1640 supplemented with 10% FBS. Tumor cells (1.5×10^6 cells per mouse) were injected *s.c.* into 8 weeks old and *Foxp3*$^{YFP\text{-}cre}$*Tcfeb*$^{fl/fl}$ and their sex-matched littermate *Foxp3*$^{YFP\text{-}cre}$ mice. Tumor volumes were measured along major

axis (a) and minor axis (b) daily and were calculated using the formula: $V=ab^2/2$. Mice were sacrificed and tumors were excised and weighted.

For TILs isolation, tumor specimens were gently minced into small pieces, then digested with 6 ml PBS containing 50 μl 25 mg/ml collagenase IV (Invitrogen) and 25 μl 10 mg/ml DNase I (Roche) for 1 h at 37°C. Cell suspensions were filtered twice and centrifuged at 1500 rpm for 5 min. Tumor cells and TILs were enriched and harvested separately by Percoll gradient (GE healthcare) following the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Statistical significance was determined by Student's *t* test or for variances by ANOVA. $p < 0.05$ was considered significant. Data are presented as Means ± SEM.

Figure S1

(**A**) Confocal microscopy analysis of TFEB in spleens from given ad libitum access to normal chow diet or starved C57/BL6 mice for 24 h. Scale bars, 20 μm. (**B**) Flow cytometric analysis (left) and quantitative results of geometric MFI (right) of p-S6 in splenocytes sorted from untreated or starved C57/BL6 mice. (**C**) Flow cytometric analysis (left) and quantitative results of $CD4^+Foxp3^+$ Treg cells in the peripheral blood of untreated or starved C57/BL6 mice for 48 h (n=5). (**D**) Immunoblot analysis of cytoplasmic and nuclear TFEB expression in TCR-activated $CD4^+$ T cells in the presence of anti-mIL-2 and varying amounts of hIL-2 for 12 h. $*p < 0.05$, $***p < 0.001$. Data are means ± SEM and were analyzed by two-tailed, unpaired Student's *t*-test.

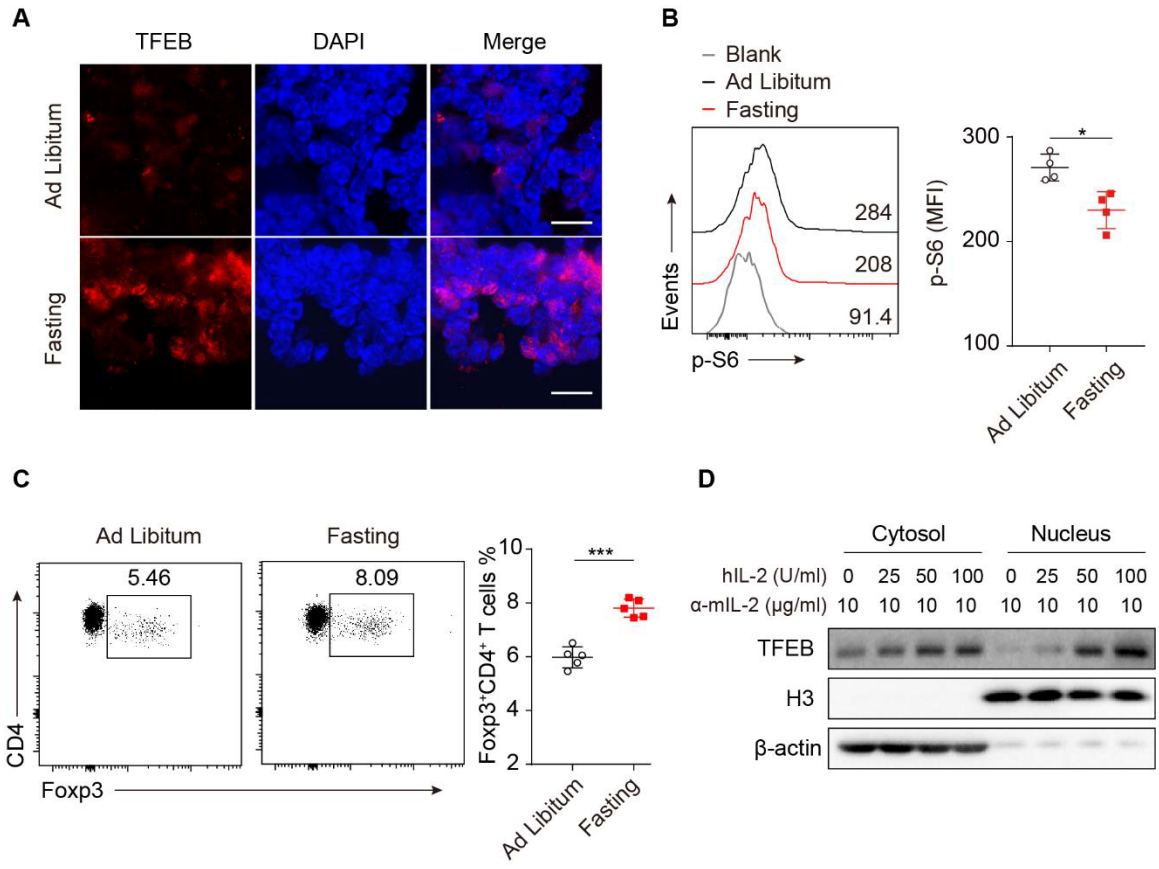


Figure S2

(**A**) Real-time PCR analysis of *Tcfeb* mRNA expression in $CD4^+$ T cells isolated from $Tcfeb^{fl/fl}$ mice and $Cd4^{Cre}Tcfeb^{fl/fl}$ mice. (**B**) Immunoblot analysis of TFEB protein expression in $CD4^+$ T cells isolated from $Tcfeb^{fl/fl}$ mice and $Cd4^{Cre}Tcfeb^{fl/fl}$ mice. (**C**) Confocal microscopy analysis of TFEB (Red) in spleens from $Cd4^{Cre}Tcfeb^{fl/fl}$ mice. Scale bars, 10 μm. (**D, E**) $CD4^+Foxp3^+NRP1^+$ tTregs (D) and $CD4^+Foxp3^+NRP1^-$ pTregs (E) in spleen, pLNs and colonic LP of 20-wk-old $Tcfeb^{fl/fl}$ and $Cd4^{Cre}Tcfeb^{fl/fl}$ mice (n=4). (**F-H**) Total cell numbers in spleen and pLNs (F) Representative plots and frequencies of $CD44^{hi}CD62L^{low}CD4^+$ or $CD44^{hi}CD62L^{low}CD8^+$ T cells in splenocytes (G), and IFN-γ-producing $CD4^+$ or $CD8^+$ T cells in splenocytes (H) from 20-wk-old $Tcfeb^{fl/fl}$ mice and $Cd4^{Cre}Tcfeb^{fl/fl}$ mice (n=4). Data are representative of four (A, B) experiments. Data are means ± SEM and were analyzed by two-tailed, Student's *t*-test.

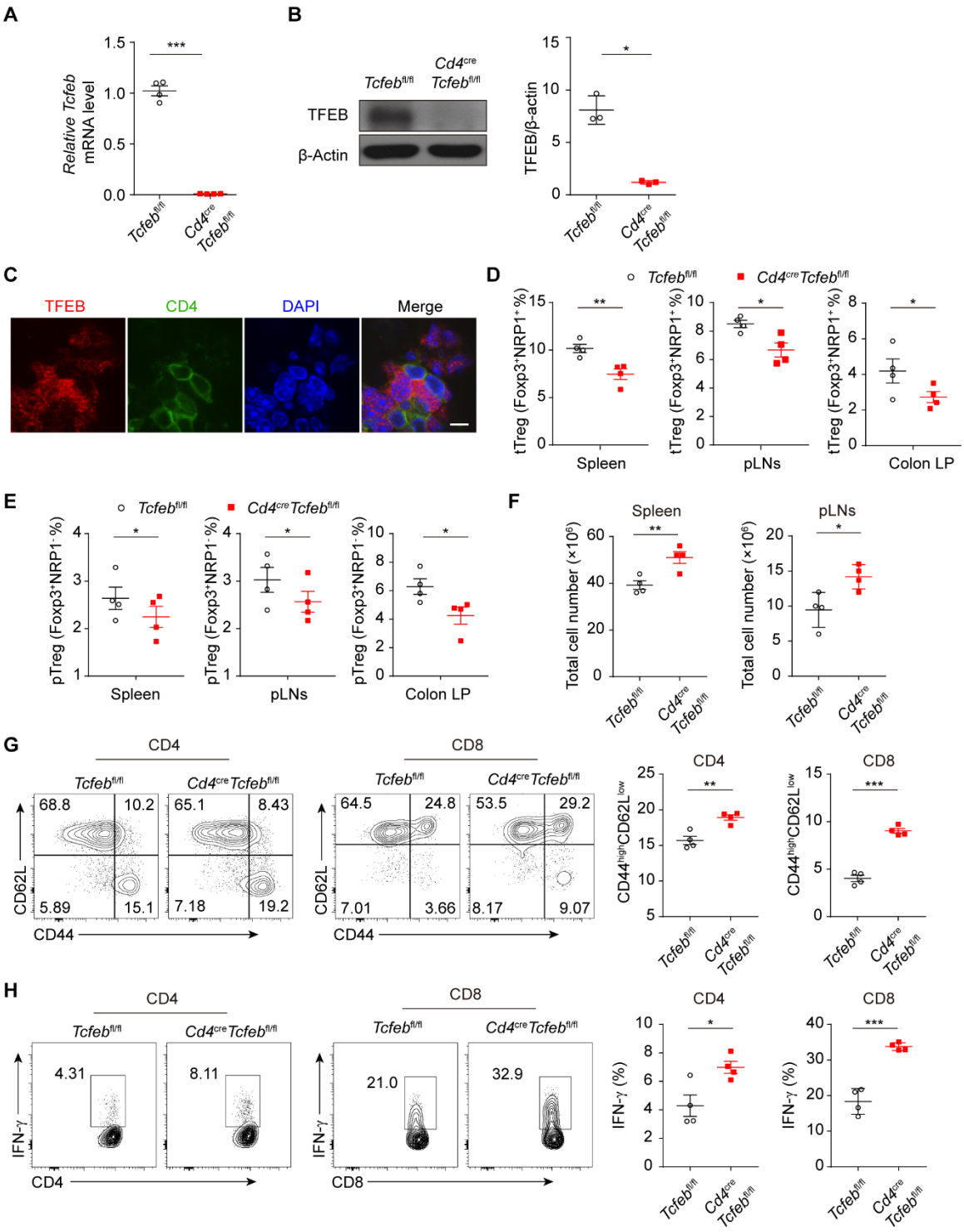

A
Relative Tcfeb mRNA level
Tcfeb fl/fl
Cd4cre Tcfeb fl/fl
B
TFEB
β-Actin
TFEB/β-actin
C
TFEB
CD4
DAPI
Merge
D
tTreg (Foxp3+NRP1+ %)
Spleen
pLNs
Colon LP
E
pTreg (Foxp3+NRP1- %)
F
Total cell number (×10^6)
G
CD4
CD8
CD62L
CD44
CD44highCD62Llow
H
IFN-γ
IFN-γ (%)

Figure S3

(**A, B**) Total cell numbers (**A**) and frequencies of Treg cells (**B**) in spleen and pLNs of 8-wk-old *Foxp3*$^{YFP\text{-}Cre}$ mice or *Foxp3*$^{YFP\text{-}Cre}$*Tcfeb*$^{fl/fl}$ mice (n=6). (**C**) Spleen weights in *Rag2*$^{-/-}$ mice receiving CD4^{+}CD25^{-}CD45RB^{high} T cells alone or in combination with either WT or *Tcfeb*-knockout CD4^{+}CD25^{+}YFP^{+} Treg cells (n=5). (**D**) Flow cytometric analysis (left) and frequencies and numbers (right) of IL-17 and IFN-γ-producing CD4^{+} T cells in the mesenteric LNs from recipient mice at 12 weeks after adoptive transfer (n=5). *$p < 0.05$. Data are representative of two independent experiments with similar results. Data are means ± SEM and were analyzed by two-tailed, unpaired Student's *t*-test.

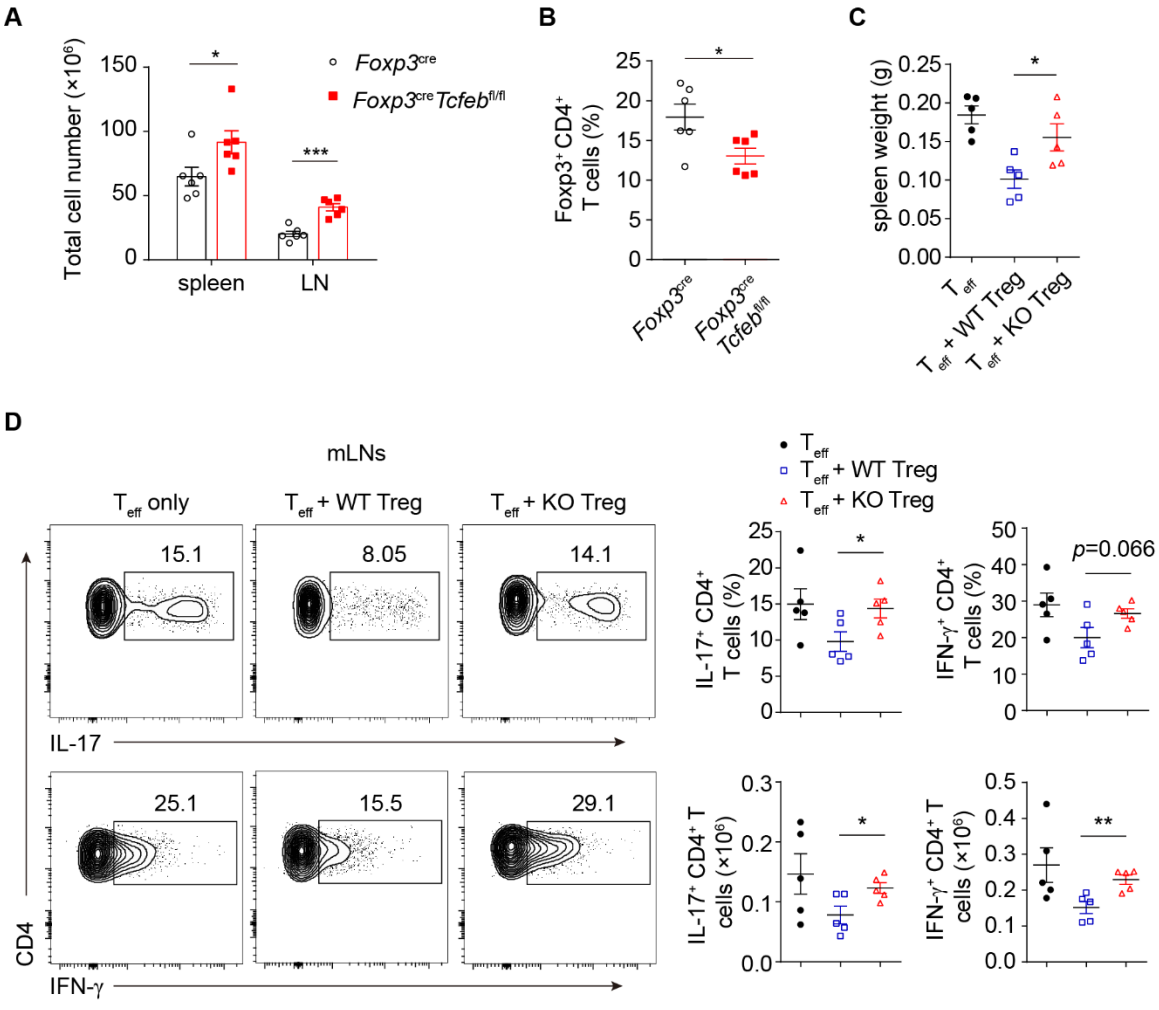


Figure S4

(**A**) Flow cytometry gating strategy of *Foxp3*$^{YFP\text{-}Cre}$ ($CD45.1^+CD45.2^+$) and *Foxp3*$^{YFP\text{-}Cre}$*Tcfeb*$^{fl/fl}$ ($CD45.1^-CD45.2^+$) Treg cells in chimera. (**B**) MFI of Foxp3 of wild-type ($CD45.1^+CD45.2^+$ *Foxp3*$^{YFP\text{-}Cre}$) and *Tcfeb*-deficient ($CD45.1^-CD45.2^+$ *Foxp3*$^{YFP\text{-}Cre}$*Tcfeb*$^{fl/fl}$) $CD4^+Foxp3^+$ Treg cells recovered in the spleens, pLNs and colonic lamina propria (LP) from chimera (n=10 or 11). (**C**) Naive $CD4^+$ T cells isolated from *Foxp3*$^{YFP\text{-}Cre}$ and *Foxp3*$^{YFP\text{-}Cre}$*Tcfeb*$^{fl/fl}$ mice were differentiated under Treg conditions for 3 days, and then YFP^+ were sorted and cultured with plate-bound anti-CD3/anti-CD28 (1 µg/ml of each) for 48 h. Representative flow cytometry plots and frequencies of Foxp3-positive cells were determined. (**D**) Representative histograms and quantitative MFI of BCL2 in wild-type and *Tcfeb*-deficient Treg cells splenocytes of chimera (n=11). *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$; Data are means ± SEM and were analyzed by two-tailed, paired Student's *t*-test.

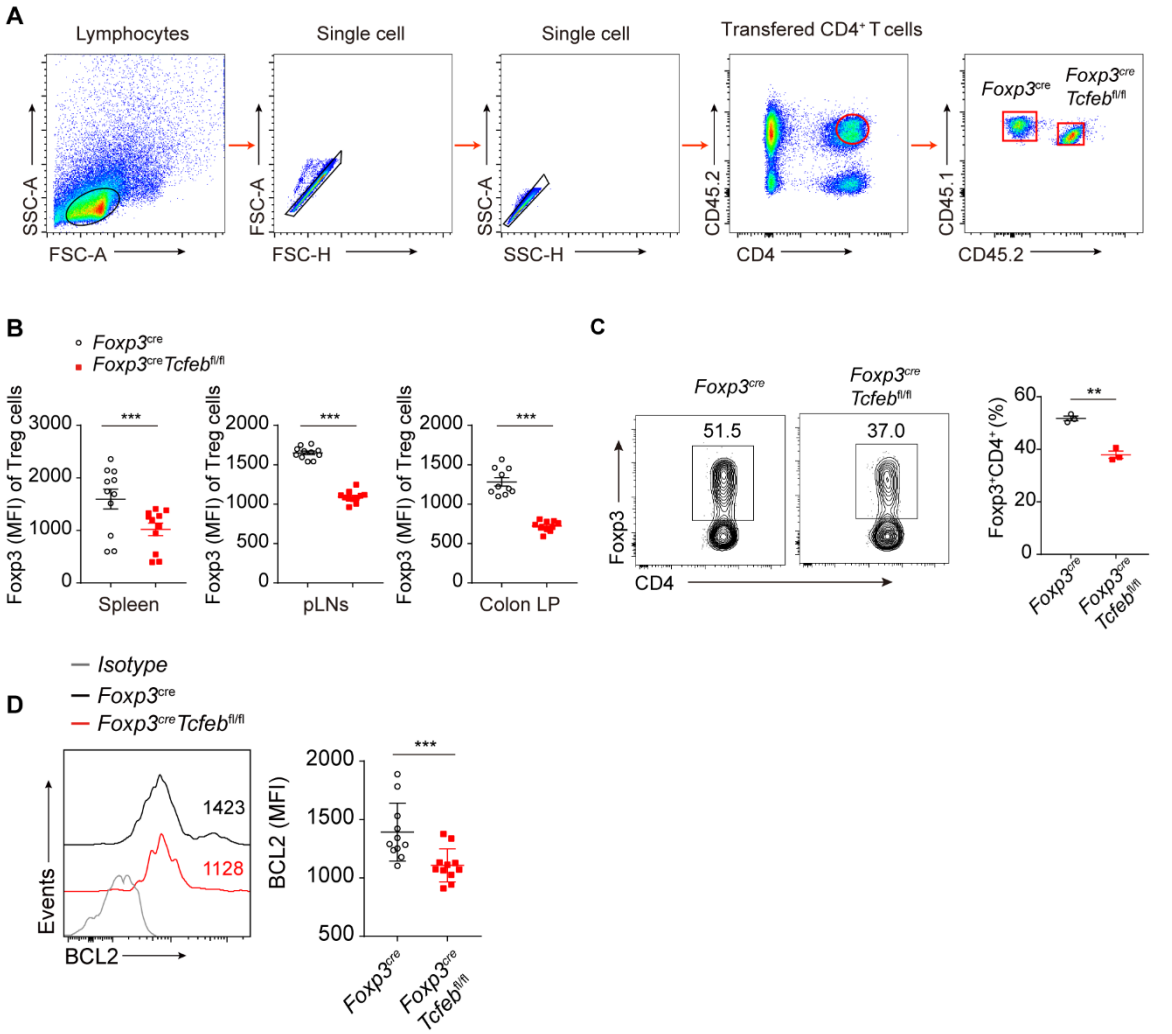


Figure S5

(**A**, **B**) WT or *Tcfeb*-deficient naive $CD4^+$ T cells were differentiated under Treg condition in the absence or presence of rapamycin (100 nM) for 60 h. The expressions of autophagy related genes (A) and lysosome related genes (B) were determined by RT-PCR. (**C**) Flow cytometric analysis (left) and quantitative MFI (right) of lysotracker in Treg cells differentiated from naïve $CD4^+$ T cells isolated from $Tcfeb^{fl/fl}$ mice and $Cd4^{Cre}Tcfeb^{fl/fl}$ mice. (**D**) WT or *Tcfeb*-deficient naive $CD4^+$ T cells were differentiated under Treg condition for 3 days in the absence or presence of rapamycin (100 nM) or BafA1 (100 nM). Protein expression was determined by immunoblot and quantified. (**E, F**) WT or *Tcfeb*-deficient naive $CD4^+$ T cells were differentiated under Treg conditions, in the presence of rapamycin (100 nM), BafA1 (100 nM) and combination of rapamycin and BafA1 for 3 days. (E) Representative flow cytometry plots and (F) frequencies of $CD4^+Foxp3^+$ Treg cells were determined. (**G**) WT or *Tcfeb*-deficient naive $CD4^+$ T cells were differentiated under Treg conditions, in the presence of rapamycin (100 nM) and 3-MA (5 mM) for 3 days. LC-3 and p62 expression were determined by western blot. ns, no significance $*p < 0.05$, $**p < 0.01$, $*** p < 0.001$; Data are means ± SEM and were analyzed by two-tailed, paired Student's t-test (C) and two-way ANOVA (D, F).

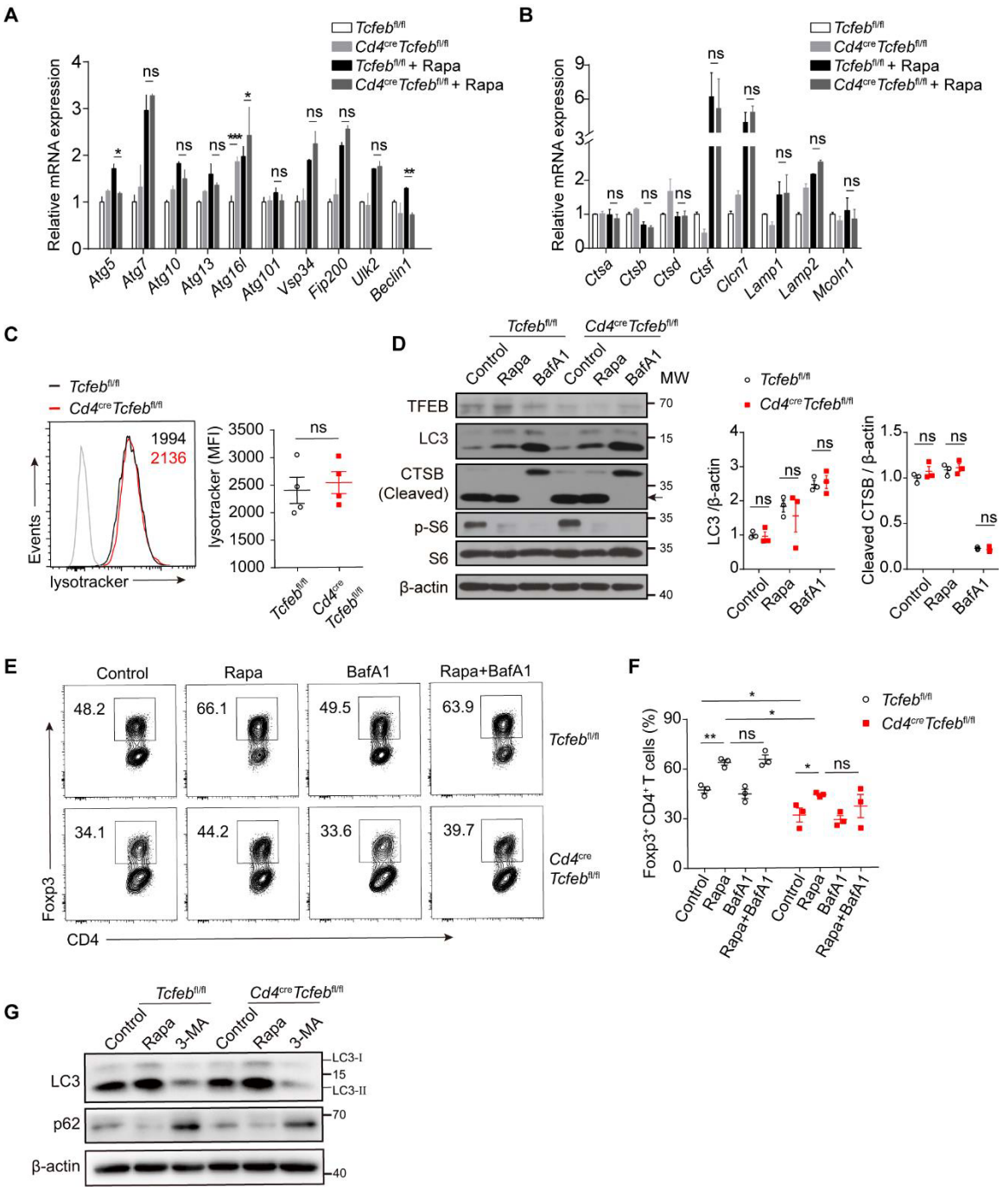
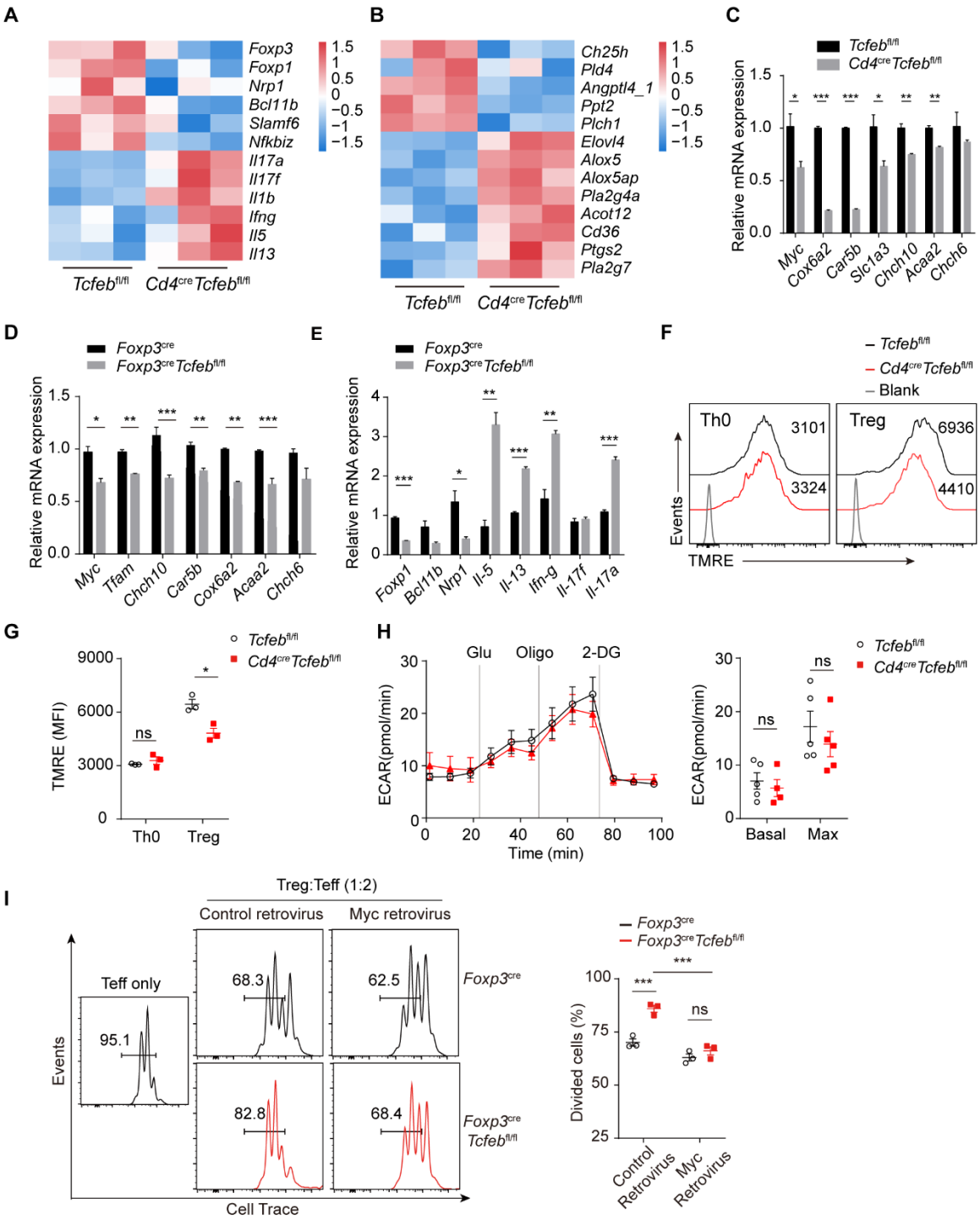

A
Tcfeb fl/fl
Cd4cre Tcfeb fl/fl
Tcfeb fl/fl + Rapa
Cd4cre Tcfeb fl/fl + Rapa
Relative mRNA expression
Atg5
Atg7
Atg10
Atg13
Atg16l
Atg101
Vsp34
Fip200
Ulk2
Beclin1
B
Ctsa
Ctsb
Ctsd
Ctsf
Clcn7
Lamp1
Lamp2
Mcoln1
C
Events
lysotracker
1994
2136
lysotracker (MFI)
D
Control
Rapa
BafA1
MW
TFEB
LC3
CTSB (Cleaved)
p-S6
S6
β-actin
LC3 /β-actin
Cleaved CTSB / β-actin
E
Control
Rapa
BafA1
Rapa+BafA1
48.2
66.1
49.5
63.9
34.1
44.2
33.6
39.7
Foxp3
CD4
F
Foxp3+ CD4+ T cells (%)
G
3-MA
LC3
p62
β-actin
LC3-I
LC3-II

Figure S6

(**A, B**) Heatmap showing the expression patterns of Treg-related genes and fatty acid metabolism related genes in WT and TFEB-deficient iTregs. The color density indicates the expression of genes, each row was scaled by z-score. (**C**) Real-time PCR analysis of mitochondria-related genes were shown. (**D, E**) Naïve $CD4^+$ T cells isolated from *Foxp3*$^{YFP\text{-}Cre}$ and *Foxp3*$^{YFP\text{-}Cre}$*Tcfeb*$^{fl/fl}$ mice were differentiated under Treg conditions for 3 days, and then YFP^+ WT and TFEB-deficient Treg cells were sorted by flow cytometry. Sorted YFP^+ Treg cell were used for RT-PCR analysis of mitochondria-related genes (D) and immune-related genes (E). (**F, G**) Representative histogram (F) and MFI quantification (G) of TMRE in WT and *Tcfeb*-deficient Th0 or Treg cells. (**H**) Extracellular acidification rate (ECAR) of WT and *Tcfeb*-deficient Treg cells were shown. (**I**) $CD4^+YFP^+$ Treg cells sorted from *Foxp3*$^{YFP\text{-}Cre}$ mice or *Foxp3*$^{YFP\text{-}Cre}$*Tcfeb*$^{fl/fl}$ mice were transduced with *Myc*-encoding retrovirus or control retrovirus. After transduction, Treg cells were co-cultured with CTV (Cell Trace Violet)-labeled WT naïve $CD4^+$ T cells at 1:2 ratio in the presence of plate-bound anti-CD3/anti-CD28 (1 μg/ml of each). The proliferative ability of T effector cells were determined by divided cells in the histograms. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Data are representative of two experiments (H, I). Data are means ± SEM and were analyzed by two-tailed, unpaired Student's *t*-test (C-H) and two-way ANOVA (I).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Foxp3
Foxp1
Nrp1
Bcl11b
Slamf6
Nfkbiz
Il17a
Il17f
Il1b
Ifng
Il5
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Cd4cre Tcfeb fl/fl
B
Ch25h
Pld4
Angptl4_1
Ppt2
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Elovl4
Alox5
Alox5ap
Pla2g4a
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Ptgs2
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C
Relative mRNA expression
Myc
Cox6a2
Car5b
Slc1a3
Chch10
Acaa2
Chch6
D
Foxp3cre
Foxp3cre Tcfeb fl/fl
Relative mRNA expression
Myc
Tfam
Chch10
Car5b
Cox6a2
Acaa2
Chch6
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Foxp3cre Tcfeb fl/fl
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Il-13
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Il-17a
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Blank
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3324
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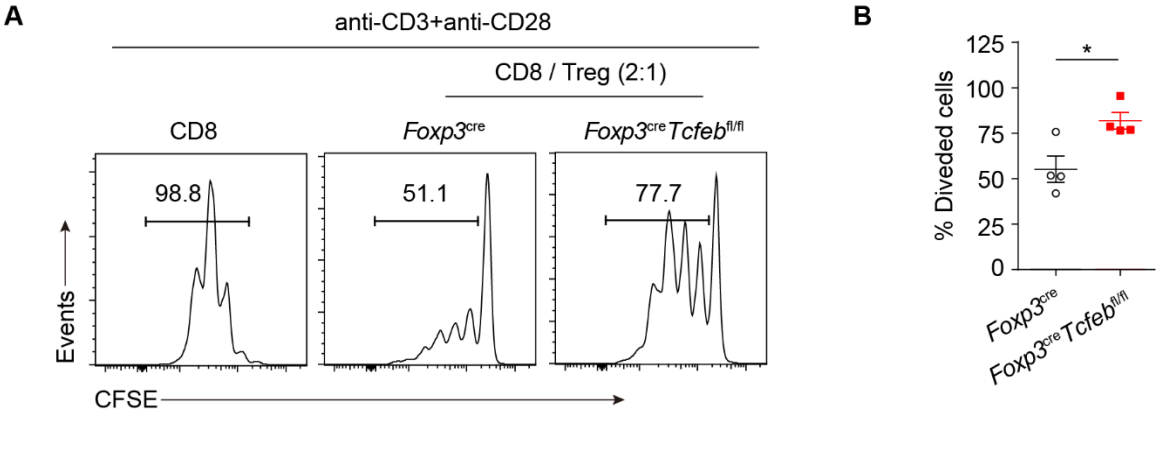
Events
TMRE
G
Tcfeb fl/fl
Cd4cre Tcfeb fl/fl
TMRE (MFI)
ns
Th0
Treg
H
Glu
Oligo
2-DG
ECAR(pmol/min)
Time (min)
Basal
Max
I
Treg:Teff (1:2)
Control retrovirus
Myc retrovirus
Teff only
95.1
68.3
62.5
82.8
68.4
Foxp3cre
Foxp3cre Tcfeb fl/fl
Events
Cell Trace
Divided cells (%)
Control Retrovirus
Myc Retrovirus

Figure S7

(**A, B**) CFSE-labeled splenic $CD8^+$ T cells stimulated with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) were co-cultured with indicated ratios of YFP^+ Treg cells sorted from *Foxp3*$^{YFP\text{-}cre}$ mice and *Foxp3*$^{YFP\text{-}cre}$*Tcfeb*$^{fl/fl}$ mice for 72 h. Flow cytometric analysis (A) and quantitative results (B) were shown. * $p < 0.05$. Data are representative of four experiments. Data are means ± SEM and were analyzed by two-tailed, unpaired Student's *t*-test.



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