Science Immunology
AAAS

Supplementary Materials for

TRAF6 prevents fatal inflammation by homeostatic suppression of MALT1 protease

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Sci. Immunol. **6**, eabh2095 (2021)
DOI: 10.1126/sciimmunol.abh2095

The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Supplementary Materials and Methods

Antibodies for immune cell phenotyping

Staining was performed with anti-CD3-PECy7 (1:300, 25-0031-82, RRID: AB_469572), anti-CD45R-PerCP-Cy5.5 (1:200, Biolegend, 103234, AB_893353), anti-CD8a-FITC (1:100, 11-0081-85, RRID: AB_464916), anti-CD4-PE (1:300, 12-0042-85, RRID: AB_465512), anti-CD4-PerCP-Cy5.5 (1:300, 45-0042-82, RRID: AB_1107001), anti-CD44-PECy7 (1:400, 25-0441-82, RRID: AB_469623), anti-CD44-FITC (1:300, 11-0441-81, RRID: AB_465044), anti-CD62L-APC (1:300, BD Pharmingen, 553152, RRID: AB_398533), anti-CD86-FITC (1:100, 11-0862-82, RRID: AB_465148), anti-CD69-APC (1:200, 17-0691-82, RRID: AB_1210795), anti-Ox40-PECy7 (1:200, 25-1341-80, RRID: AB_2573395), anti-CTLA-4-PECy7 (1:200, Biolegend, 106313, RRID: AB_2564237), anti-CD19-APC (1:200, Elabscience, E-AB-F0986UE), anti-CD23-Biotin (1:300, 13-0232-81, RRID: AB_466392), anti-Strepdavadin-APC (1:100, 17-4317-82), anti-CD21-FITC (1:300, BD Pharmingen, 561769, RRID: AB_10924591), anti ICOS-FITC (1:200, 11-9949-82, RRID: AB_465458) and anti-IκNS (1:10, 4C1, HMGU). All antibodies are from eBiosciences except where indicated. For intracellular staining of FoxP3, cells were permeabilized and fixed (eBiosciences, 00-5223-56), washed once with permeabilization buffer (eBiosciences, 00-8333-56) and stained for 30 min with anti-FoxP3-PE (1:100, eBiosciences, 12-5773-82, RRID: AB_465936) in permeabilization buffer. Cells were washed with sorting buffer and analyzed using an Attune Acoustic Focusing Cytometer (Thermo Fisher) or a Fortessa Cytometer (Becton Dickinson, Franklin Lakes, NJ).

Cultivation, stimulation and inhibitor treatment of cell lines and primary cells

All cell lines were maintained in humidified atmosphere (37°C, 5% CO_2). Jurkat T cells were cultured in RPMI 1640 Medium and maintained at a density between 0.5x and 1.5x 10^6 cells/ml. HEK293T cells were kept in DMEM and diluted after treatment with 0.05% trypsin/EDTA solution upon reaching a confluency of more than 80%. Media were supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin (all Gibco). Jurkat T cells were verified by the Authentication Service of the Leibniz Institute

(DSMZ). HEK293T cells were obtained from and verified by DSMZ (RRID: CVCL_0045/CVCL_0063). Primary murine splenocytes were isolated from spleen and treated with Red Blood Cell Lysis Solution (Miltenyi), and $CD4^+$ T cells were purified using a $CD4^+$ T cell isolation kit II (Miltenyi) by negative magnetic-activated cell sorting (MACS). $CD4^+$ T cells were cultured in primary T cell medium (RPMI 1640, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% heat inactivated fetal calf serum, 10 mM HEPES pH 7.5, 2 mM L-Glutamine, 1 mM Sodium-Pyruvate, MEM-NEAA (1x), 50 nM β-Mercaptoethanol (all Gibco). Jurkat T cells were stimulated with Phorbol 12-Myristate 13-Acetate (PMA, 200 ng/ml; Merck)/Ionomycin (Iono, 300 ng/ml; Calbiochem), mouse anti-human CD3 (1µg/ml; BD Pharmingen #555336; RRID: AB_395742)/CD28 (3.3µg/ml; BD Pharmingen #555725; RRID: AB_396068) in presence of rat anti-mouse IgG1 (1.65µg/ml; BD Pharmingen #553440; RRID: AB_394860) and IgG2a (1.65µg/ml; BD Pharmingen #553387; RRID: AB_394825) (all BD Pharmingen) or recombinant human TNFα (20 ng/ml, Biomol). $CD4^+$ T cells were stimulated with PMA (200 ng/ml)/Iono (300 ng/ml) or hamster anti-mouse CD3 (0.5 µg/ml; 145-2C11; BD Bioscience #557306; RRID: AB_396632)/CD28 (1 µg/ml; 37.51; BD Bioscience #553295; RRID: AB_394764) on rabbit anti-hamster IgG (Jackson ImmunoResearch #307-005-003; RRID: AB_2339572) pre-coated plates. For *in vitro* treatment of Jurkat T cells with MLT-943 (*28*) or MLT-985 (*37*), cells were seeded in 6-well plates and incubated with the respective inhibitor concentrations for 24 hours in humidified atmosphere.

Analyses of IκBNS and ICOS expression by flow cytometry

For intracellular IκBNS and extracellular ICOS staining, primary murine splenocytes ($1x10^6$) were collected, centrifuged (300 x g, 5 min, 4 °C) and washed twice with PBS. Live/dead staining (Fixable Viability Dye eFluor 780, 1:1000 in PBS, eBioscience #65-0865-14) was added for 30 min at 4°C. Cells were washed with PBS, fixed in 2 % PFA for 20 min at 4 °C and permeabilized in Saponin buffer (0,5 % saponin and 1% BSA in PBS) for 25 min at RT. Unspecific antibody binding was blocked with anti-CD16/32 (1:100 in Saponin buffer, 10 min, 4 °C, eBioscience #14-0161-81, RRID: AB_467132) and

samples were incubated with anti-IκBNS antibody (clone 4C1, rat, 1:10 in Saponin buffer, HMGU core facility monoclonal antibodies, RRID: N/A) for 30 min at 4 °C. Cells were washed (300 x g, 5 min, 4 °C) and stained with a secondary mouse anti-rat-AF647 (1:200 in Saponin buffer, 30 min at 4 °C, Biolegend #405416, RRID: AB_2562967). In parallel, anti-ICOS-FITC antibody in Saponin buffer was added (eBiosciences #11-9949-82, RRID: AB_465458). Samples were washed (300 x g, 5 min, 4 °C) and wells were filled up with Saponin buffer to wash out unbound antibodies (> 15 min, RT). For surface staining, anti-CD4-PerCP (eBiosciences #45-0042-82, RRID: AB_1107001) and anti-CD8-PE (BD Pharmingen #553033, RRID: AB_394571) was added for 30 min at RT. Samples were washed with FCM buffer (300 x g, 5 min, 4 °C), re-suspended in FCM buffer and acquired on an Attune Cytometer.

Preparation of tissue sections and histopathological evaluation

Tissue from mice was fixed in 10% neutral buffered formalin, embedded in paraffin (both Thermo Fisher Scientific, Waltham, Massachusetts, United States of America), cut at 4 µm using a microtome (Leica Biosystems, Nussloch GmbH, Nussloch, Germany), mounted onto glass slides (Thermo Fisher Scientific) and stained by Hematoxylin and Eosin (Thermo Fisher Scientific) on an automatic stainer (AutostainerXL, Leica Biosystems) using standard protocols. Glass slides were scanned at 400x (AperioCS2, Leica Biosystems) and subsequently evaluated using an open source software for digital visualization and analysis (QuPath, v.0.2.3, University of Edinburgh, Scottland, United Kingdom) (*58*). Representative images were exported using the same software.

***In vitro* Treg cell suppression assay**

Treg suppression assays were performed as described previously (*21*). $CD4^+CD25^-CD45RB^{hi}$ conventional naïve T cells and $CD4^+CD25^-CD45RB^{lo}$ Treg cells were sorted with a FACS Aria III (BD Biosciences). Conventional $CD4^+$ T cells were labeled with 10 µM Cell Proliferation Dye eFluor 450 (Thermo Fisher Scientific) according to the manufacturer's protocol. 2.5×10^4 labeled conventional naïve $CD4^+$ T cells

were plated alone or in presence of varying numbers of Treg cells in RPMI-1640/10% FBS complete medium into a 96-well U-bottom plate. Following the addition of 1 μg mL^{-1} (final concentration) of soluble anti-CD3 clone 17A2 (eBioscience, 100201, RRID: AB_312658) and 5×10^4 splenocytes that had been irradiated with 30 Gy using a BIOBEAM 3000 gamma irradiation device, cells were incubated for 3 days at 37 °C and 5% CO_2. Subsequently, the cells were subjected to flow cytometric analysis on a FACSCanto II (BD Biosciences).

Preparation of cell lysates

For cellular analysis via WB, Jurkat or primary murine $CD4^+$ T cells (2-3x10^6) were washed 1x in PBS and lysed in co-immunoprecipitation (co-IP) buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 0.2% NP-40, 10% glycerol, 1 mM DTT, 10 mM NaF, 8 mM β-glycerophosphate, 300 μM sodium vanadate and protease inhibitor cocktail mix (Roche)) for 20 min at 4°C. For cellular lysis of EMSA samples, a high salt buffer was used (20 mM HEPES pH 7.9, 350 mM NaCl, 20% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP-40, 1 mM DTT, 10 mM sodium fluoride, 8 mM β-glycerophosphate, 300 μM sodium vanadate and Roche protease inhibitor cocktail mix). Lysate controls were mixed with 4x SDS loading dye, boiled for five min at 95°C, separated by SDS-PAGE and analyzed by WB.

Western blotting (WB)

An electrophoretic semi-dry blotting system was used to transfer SDS-PAGE separated proteins onto PVDF-membranes (Merck Millipore). After transfer, membranes were blocked with 5% BSA (Sigma-Aldrich) or 5% milk (Roth) in PBS-Tween (0.01% Tween) for 1 hour at RT. Primary antibodies were diluted as indicated in 2.5% BSA or milk in PBS-T and membranes incubated overnight at 4°C. Membranes were washed 3x 15 min with PBS-T and treated with HRP-coupled secondary antibodies (1:7000 in 1.25% BSA or milk in PBS-T) for 1 hour at RT. HRP was detected by enhanced chemiluminescence using the LumiGlo reagent kit (Cell Signaling Technologies) according to the manufacturer's specifications and

visualized on ECL Amersham Hyperfilms (GE Healthcare). Images were cropped for presentation. WB antibodies used: anti-CARD11 (1D12; #4435; RRID: AB_10694496), anti-IκBα (L35A5; #4814; RRID: AB_390781), anti-p-IκBα (Ser32/36, 5A5; #9246; RRID: AB_2151442), anti-p65 (D14E12; #8242; RRID: AB_10859369), anti-p-p65 (93H1; #3033; RRID: AB_331284), anti-E-tag (13419; #13419; RRID: AB_2798215), anti-p-Akt (#9271; RRID: AB_329825), anti-Akt (#9272; RRID: AB_329827) (all Cell Signaling Technology); anti-MALT1 (B-12 for human; #sc-46677; RRID: AB_627909) (D-1 for murine; #sc-515389; RRID: N/A), anti-β-Actin (C4, 1:20.000; #sc-47778; RRID: AB_2714189), anti-CYLD (E-10; #sc-74435; RRID: AB_1122022), anti-HOIL-1 (H-1; #sc-393754; RRID: N/A), anti-BCL10 (H-197; #sc-5611; RRID: AB_634292) (all Santa Cruz); anti-Regnase-1 (#MAB7875; RRID: N/A) (R&D); anti-TRAF6 (EP591Y; #ab33915; RRID: AB_778572) (Abcam); anti-Roquin-1/2 (clone 3F12, 1:10, HMGU core facility monoclonal antibodies); HRP-conjugated anti-rabbit (#711-035-152; RRID: AB_10015282), HRP-conjugated anti-mouse (#715-035-150; RRID: AB_2340770), HRP-conjugated anti-rat (#112-035-062; RRID: AB_2338133) (all Jackson ImmunoResearch, 1:7000); all antibodies were used at 1:1000 dilution if not otherwise stated.

Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were carried out using double stranded NF-κB (H2K fwd: 5'-GATCCAGGGCTGGGGATTCCCCATCTCCACAGG-3', H2K rev: 5'-GATCCCTGTGGAGATGGGGAATCCCCAGCCCTG-3') and OCT1 (fwd: 5'-GATCTGTCGAATGCAAATCACTAGAA-3', rev: 5'-GATCTTCTAGTGATTTGCATTCGACA-3') binding sequences which were radioactively labeled with [α-^{32}P] dATP using Klenow Fragment (NEB). Whole cell lysates (6-10 µg) were incubated for 30 min with shift-buffer (20 mM HEPES pH 7.9, 120 mM KCl, 4% Ficoll, 5 mM DTT, 10 µg BSA and 2 µg poly-dI-dC (Roche)) and radioactively labelled double stranded probes (10.000-20.000 cpm). Samples were applied on a 5% polyacrylamide gel in TBE buffer, vacuum-dried and exposed to Amersham autoradiography films (GE Healthcare).

Image stream analysis

Splenocytes were isolated from control, *Traf6*-ΔT or *Malt1* TBM-T mice, and either left untreated or stimulated with PMA (200 ng/ml)/Iono (300 ng/ml) for 30 min at 37°C. Fc receptors were blocked with anti-CD16/32 clone 93 (1:200, 14-0161-81, eBioscience, RRID: AB_467132), and cells stained with anti-CD4-APC (1:200, L3T4, 17-0041-83, eBioscience, RRID: AB_469321) for 15' at 4°C. Following fixation and permeabilization for 1 hour at RT using Foxp3/Transcription factor staining buffer set (00-5523-00, eBioscience) according to the manufacturer's protocol, cells were subjected to intracellular staining with anti-p65 (1:200, D14E12, Cell Signaling Technology #8242, RRID: AB_10859369) or anti-c-Rel (1:200, sc-71, Santa Cruz Biotechnology #sc-71, RRID: AB_2253705) for 1 hour at 4°C. After staining with a fluorescein isothiocyanate-coupled anti-rabbit IgG secondary antibody (1:200, 554020, BD Pharmingen, RRID: AB_395212) for 30 min at 4°C, 2µg/ml 4′,6-diamidino-2-phenylindole (Thermo Fischer) were applied to stain cell nuclei. The stained cell suspensions were subjected to flow cytometric analysis on an ImageStreamX Mark II (Amnis Merck Millipore). Data were analyzed using the IDEAS software (Amnis Merck Millipore) in which nuclear translocation is determined by quantification of the correlation between nuclear stain and intensity of the translocation probe, yielding a similarity score. High similarity score (high correlation) represents strong nuclear translocation, whereas low similarity scores indicate cytoplasmic localization. Histogram overlays were generated using FlowJO analysis software (BD).

Detection of active MALT1 by activity based probes (ABP)

Generation and application of biotin-labeled MALT1 activity based probes (MALT1-ABPs) has been described previously (*30*). Jurkat T cells ($3x10^7$) were washed with PBS and lysed in 600 µl co-IP buffer without protease inhibitors for 25 min at 4°C. Cleared lysates (>20.000 x g, 4°C, 10 min) were used to collect lysate control (60µl) or incubated with High Capacity Streptavidin Beads (Thermo Fisher, 12µl) for 1 hour at 4°C for pre-clearing (490µl). Beads were pelleted (1700 x g, 2 min, 4°C) and 420 µl of supernatant was mixed with biotin-labeled MALT1-ABP at a final concentration of 0.1 µM. After 50 min rotating at

RT, High Capacity Streptavidin Beads (Thermo Fisher, 15µl) were added and samples were incubated for 1-2 hours at 4°C (rotating). Beads were collected (1700 x g, 2 min, 4°C), washed 3x with co-IP buffer without protease inhibitors, resuspended in 22 µl 2xSDS loading dye, boiled at 95°C for 7 min and analyzed by WB.

Analysis of MALT1A and MALT1B splice isoforms

RNA from $1x10^7$ purified $CD4^+$ T cells were isolated by resuspending in 600 µl TRIzol and incubating 5 min at RT. 120 µl chloroform was added and samples were vortexed for 15 sec followed by 3 min incubation at RT. Samples were centrifuged at 13,000 rpm for 5 min at 4°C and the colorless upper aqueous phase was transferred to a new microtube. The same volume of 70% Ethanol was added and samples were resuspended. Further RNA purification was performed with the RNeasy Mini Kit (Qiagen) and RNA was reverse transcribed (Verso cDNA synthesis kit, Thermo Fisher). To determine endogenous MALT1 isoform A/B levels, semi-quantitative PCR with 15 ng cDNA was performed using Taq DNA Polymerase (NEB) with primers flanking exons 7 *(*ex6 fw: 5′-ACCGAGACAGTCAAGATAGC-3′; ex9/10 rev: 5′-GACTTTGTCCTTTGCCAAAGG-3′) and detecting both isoforms MALT1A (146 bp) and MALT1B (113 bp). Hydroxymethylbilane synthase (Hmbs) served as control (fw: 5′-GCGCTAACTGGTCTGTAGGG-3′; rev: 5′-TGAGGGAAAGGCAGATATGGAGG-3). PCR products were analyzed using 3% agarose gels.

Supplementary Figures and Tables

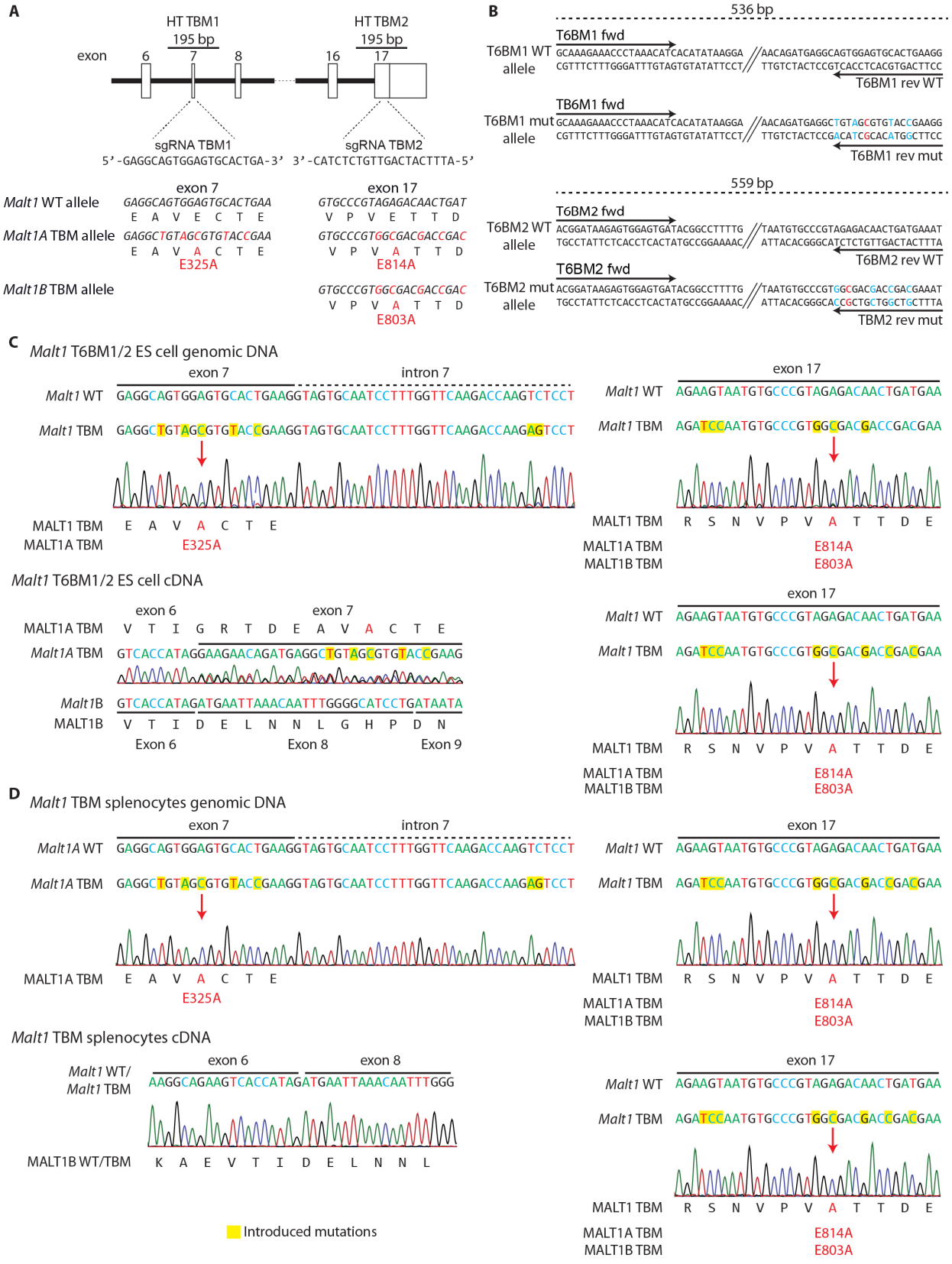


Fig. S1: Generation and verification of MALT1 TBM mutant embryonic stem (ES) cells and mice.

(A) Schematic depiction of genomic organization of murine *Malt1* exon 7 and exon 17 encoding for T6BM1 and T6BM2, respectively. Single guide (sg) RNA sequences targeting T6BM1 and T6BM2 are shown. DNA and amino acid sequences for T6BM1 (exon 7) and T6BM2 (exon 17) regions are depicted. Below, targeting strategy and alterations in the genomic DNA (including missense and silent mutations) are shown next to MALT1A E325A;E814A and MALT1B E803A protein expression. (B) Strategy for differential PCR genotyping of T6BM1 and T6BM2 wt and mutant alleles. (C and D) Sanger sequencing of genomic region and cDNA spanning T6BM1 (exon 7) and T6BM2 (exon 17) verifying the correct mutagenesis in *Malt1*$^{TBM/TBM}$ ES cells (C) and in *Malt1*$^{TBM/TBM}$ splenocytes (D). Genomic and cDNA sequencing verified correct mutations in T6BM1 and T6BM2. Note on cDNA sequences: *Malt1A* and *Malt1B* are expressed in ES cells leading to two overlapping ex6-ex7-ex8 and ex6-ex8 sequences. Correct mutations in exon7 of *Malt1A* cDNA have been verified by sequence deconvolution. *Malt1A* mRNA is not expressed in splenocytes and only sequences for *Malt1B* cDNA lacking exon7 are detected.

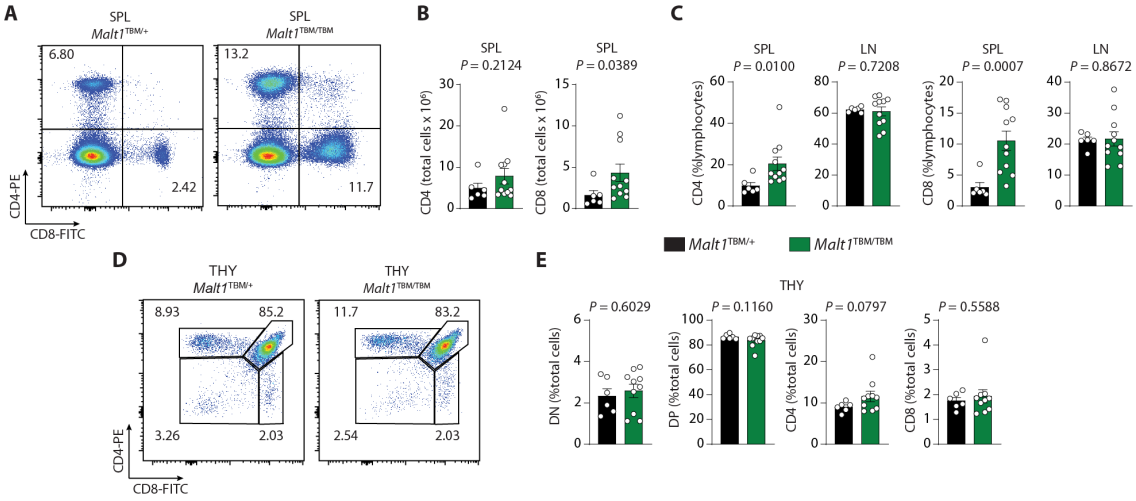


Fig. S2: Analysis of T cell numbers in *Malt1* TBM mice. (A to C) Flow cytometric analysis of $CD4^+$ and $CD8^+$ T cells (A) and quantifications showing total numbers (B) in spleen (SPL) or relative numbers (C) in spleen (SPL) and lymph nodes (LN) in *Malt1*$^{TBM/+}$ and *Malt1*$^{TBM/TBM}$ mice. (D and E) Flow cytometric analysis of $CD4^+$ and $CD8^+$ double negative (DN), double positive (DP) or single positive thymic T cells (D) and quantification of relative numbers (E) of DN, DP and single positive $CD4^+$ and $CD8^+$ T cells from thymus (THY) of *Malt1*$^{TBM/+}$ and *Malt1*$^{TBM/TBM}$ mice. All analyses were performed with animals at day 18 after birth. Heterozygous *Malt1*$^{TBM/+}$ littermates (black) were compared to *Malt1*$^{TBM/TBM}$ mice (green). Each dot represents 1 mouse (6 to 11 mice per group). All bars show the means ± SEM, and *P* values were calculated by unpaired *t* test with Welch's correction.

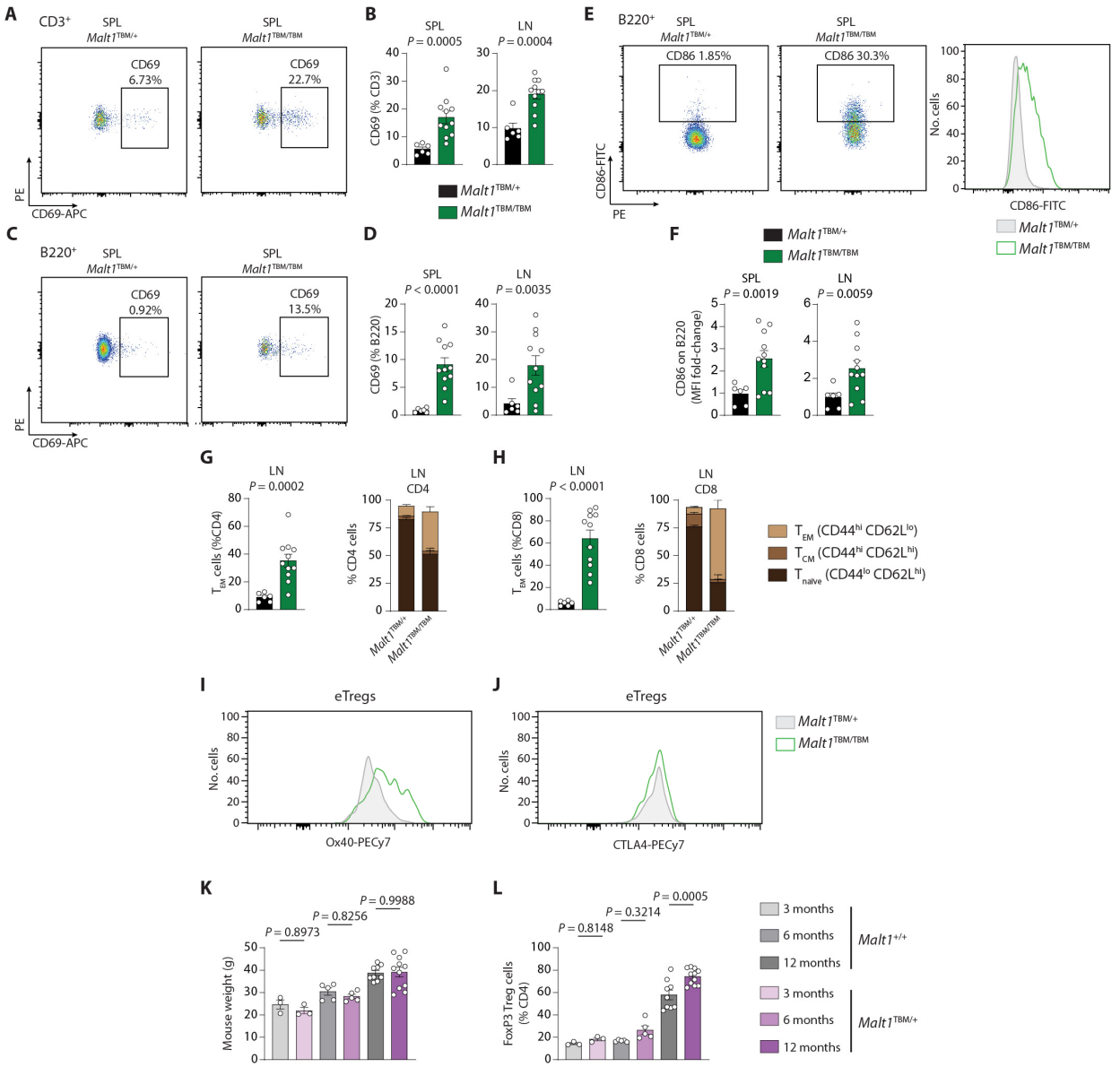


Fig. S3: Staining of lymphocyte activation and Treg cell markers in *Malt1* TBM mice. (A to D) Flow cytometric analysis of CD69 staining on $CD3^+$ (A) and $B220^+$ (C) lymphocytes. Quantification of relative numbers of $CD69^+$CD T (B) or B22 B (D) cells from spleen (SPL) and lymph nodes (LN) of *Malt1*$^{TBM/+}$ and *Malt1*$^{TBM/TBM}$ mice. (E and F) Flow cytometric analysis of CD86 staining on $B220^+$ lymphocytes (E) and fold change of CD86 mean fluorescence intensity (MFI) (F) on $B220^+$ B cells from spleen and lymph nodes between *Malt1*$^{TBM/+}$ and *Malt1*$^{TBM/TBM}$ mice. (G and H) Quantifications of relative numbers of $CD44^{hi}CD62L^{lo}$ T_{EM} cells (left) and relative numbers of $T_{naïve}$ ($CD44^{lo}CD62L^{hi}$), T_{CM} ($CD44^{hi}CD62L^{hi}$) and T_{EM} $CD4^+$ (G) and $CD8^+$ (H) T cells from lymph nodes of *Malt1*$^{TBM/+}$ and *Malt1*$^{TBM/TBM}$ mice. (I and J) Representative histograms for OX40 (I) and CTLA-4 (J) staining on $CD44^{hi}CD62L^{lo}$ effector eTreg cells from spleen of *Malt1*$^{TBM/+}$ and *Malt1*$^{TBM/TBM}$ mice. (K) Mouse weights of *Malt1*$^{+/+}$ and *Malt1*$^{TBM/+}$ mice at an age of 3, 6 or 12 months. (L) Relative numbers of $CD4^+FoxP3^+$ Treg cells in spleen of *Malt1*$^{+/+}$ and *Malt1*$^{TBM/+}$ mice at an age of 3, 6 or 12 months. All analyses (except K and L) were performed with animals at day 18 after birth and heterozygous *Malt1*$^{TBM/+}$ littermates (black) were compared to *Malt1*$^{TBM/TBM}$ mice (green). Each dot represents 1 mouse (B-H: 6 to 11 mice per group, K, L: 3 to 11 mice per group). All bars show the means ± SEM, and *P* values were calculated by unpaired *t* test with Welch's correction (in B, D, F, G and H) or by ordinary one-way analysis of variance (ANOVA) combined with Tukey's multiple comparisons test (K and L).

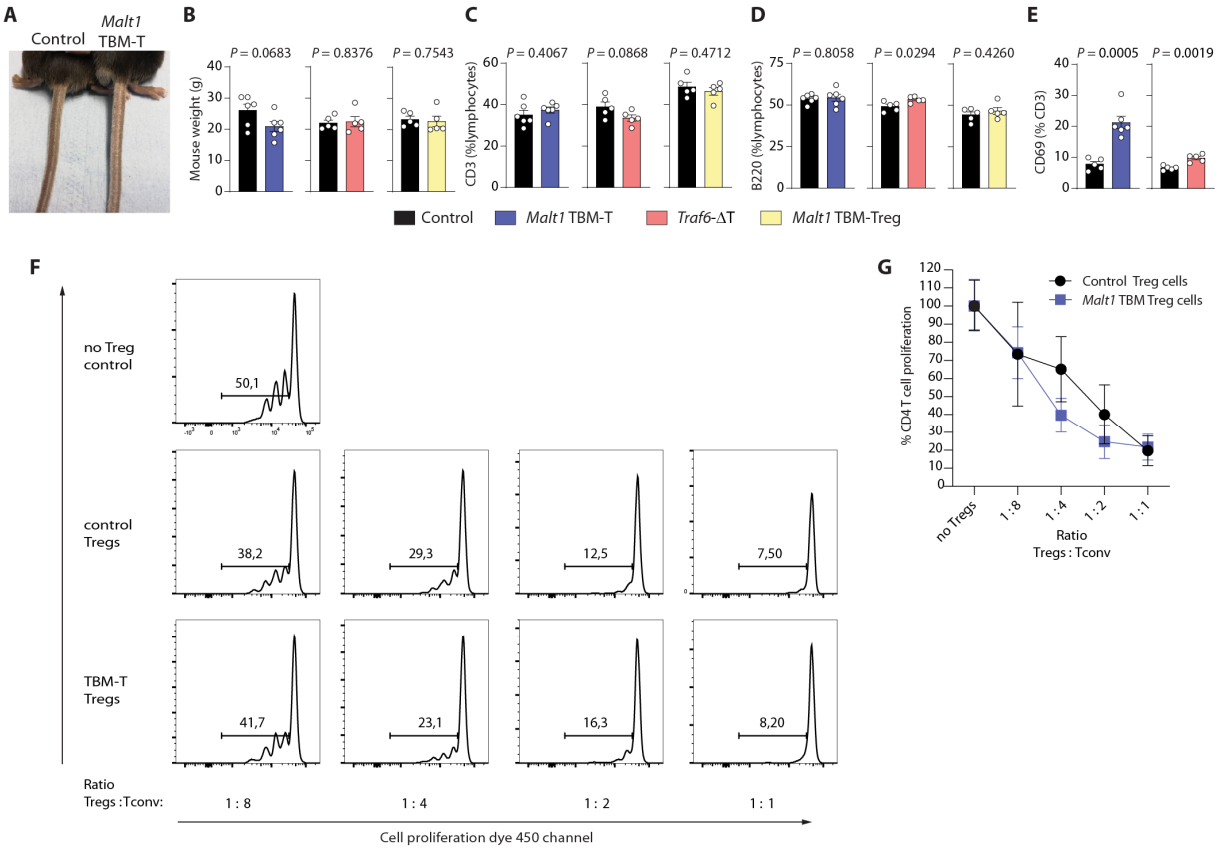


Fig. S4: Phenotypic analysis of *Malt1* TBM-T, *Traf6*-ΔT and *Malt1* TBM-Treg mice. (A) Picture of mouse tails showing skin eczema in *Malt1* TBM-T versus control mice. (B) Mouse weights of control, *Malt1* TBM-T, *Traf6*-ΔT and *Malt1* TBM-Treg mice. (C and D) Relative numbers of splenic $CD3^+$ T (C) and $B220^+$ B (D) cells in control, *Malt1* TBM-T, *Traf6*-ΔT and *Malt1* TBM-Treg mice. (E) Relative numbers of splenic $CD69^+CD3^+$ T cells from control, *Malt1* TBM-T, *Traf6*-ΔT and *Malt1* TBM-Treg mice. (F) *In vitro* Treg-suppressor assays showing representative FACS plots of proliferation dye 450 labelled conventional $CD4^+$ T cells (Tconv) cultivated without (no Tregs) or with increasing numbers of Treg cells (ratio 1:8 to 1:1) of control or *Malt1* TBM-T mice in the presence of irradiated splenocytes and αCD3. (G) Suppression of conventional $CD4^+$ T cell proliferation by control and *Malt1* TBM Treg cells (see F) was quantified by determining the percentage of $CD4^+$ T cells displaying dye 450 dilution compared to control (no Treg cells). Data were normalized to the mean Tconv proliferation in the absence of Treg cells (no Tregs) and show the means ± SD from 3 biological replicates. Two-way ANOVA with Sidak's multiple comparison correction was performed ($p>0.11$ for all ratios). All immune cell stains were performed with splenic cells. Littermate control mice were *Malt1*$^{fl/+}$*;CD4-Cre*$^-$, *Malt1*$^{fl/+}$;CD4-Cre^+, and *Malt1*$^{TBM/fl}$*;CD4-Cre*$^-$ or *Traf6*$^{fl/+}$*;CD4-Cre*$^+$, *Traf6*$^{fl/fl}$*;CD4-Cre*$^-$, and *Traf6*$^{fl/+}$*;CD4-Cre*$^-$ *or Malt1*$^{fl/+}$*;FoxP3-Cre*$^-$, *Malt1*$^{fl/+}$;FoxP3-Cre^+, and *Malt1*$^{TBM/fl}$*;FoxP3-Cre*$^-$ (black) for *Malt1* TBM-T (blue) or *Traf6*-ΔT (rose) or *Malt1* TBM-Treg (yellow), respectively. Each dot in panels B-E represents 1 mouse (5 to 6 mice per group). Treg suppression assay was performed with 3 sets of mice (control and TBM-T). All bars (B-E) show the means ± SEM, and *P* values were calculated by unpaired *t* test with Welch's correction.

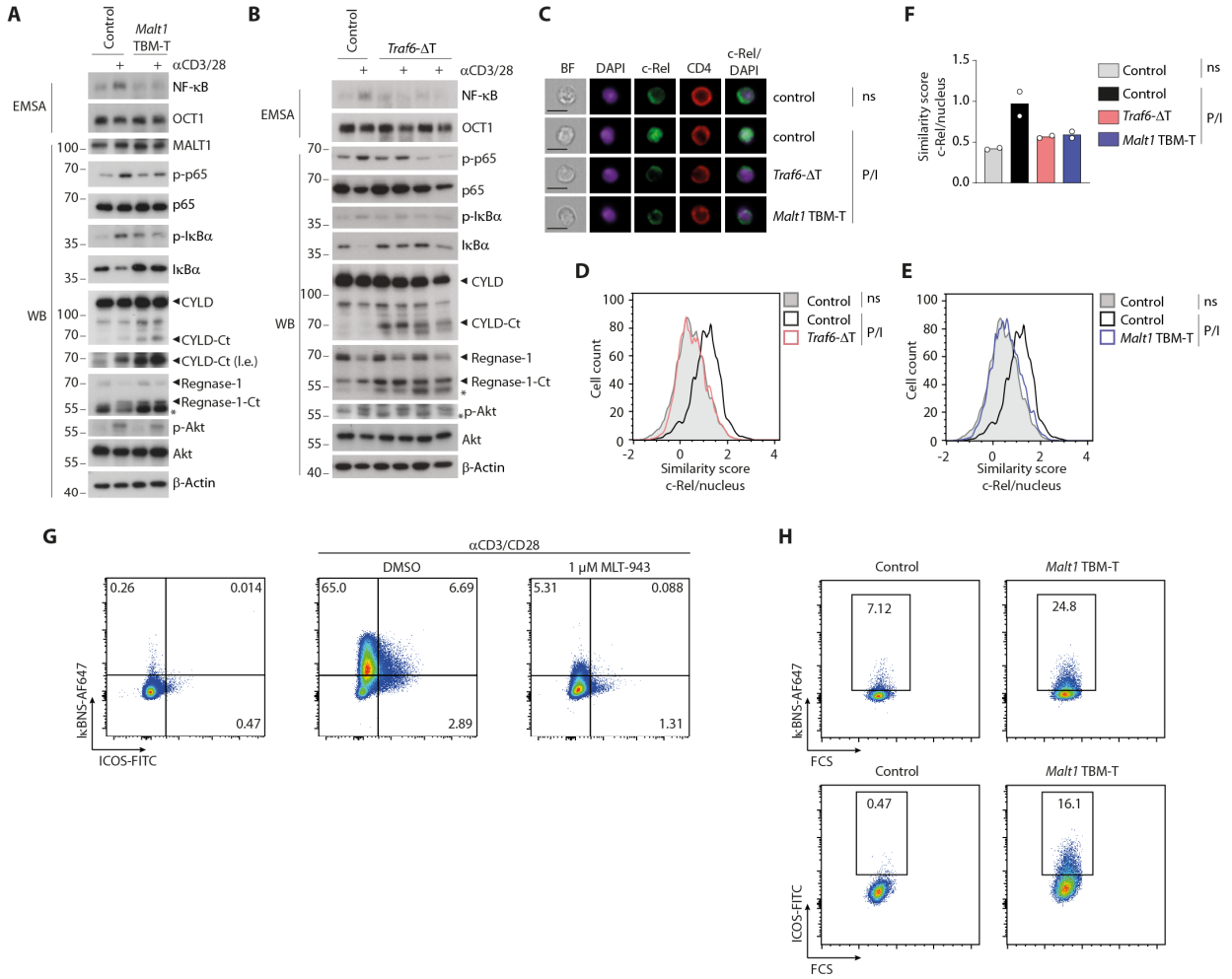
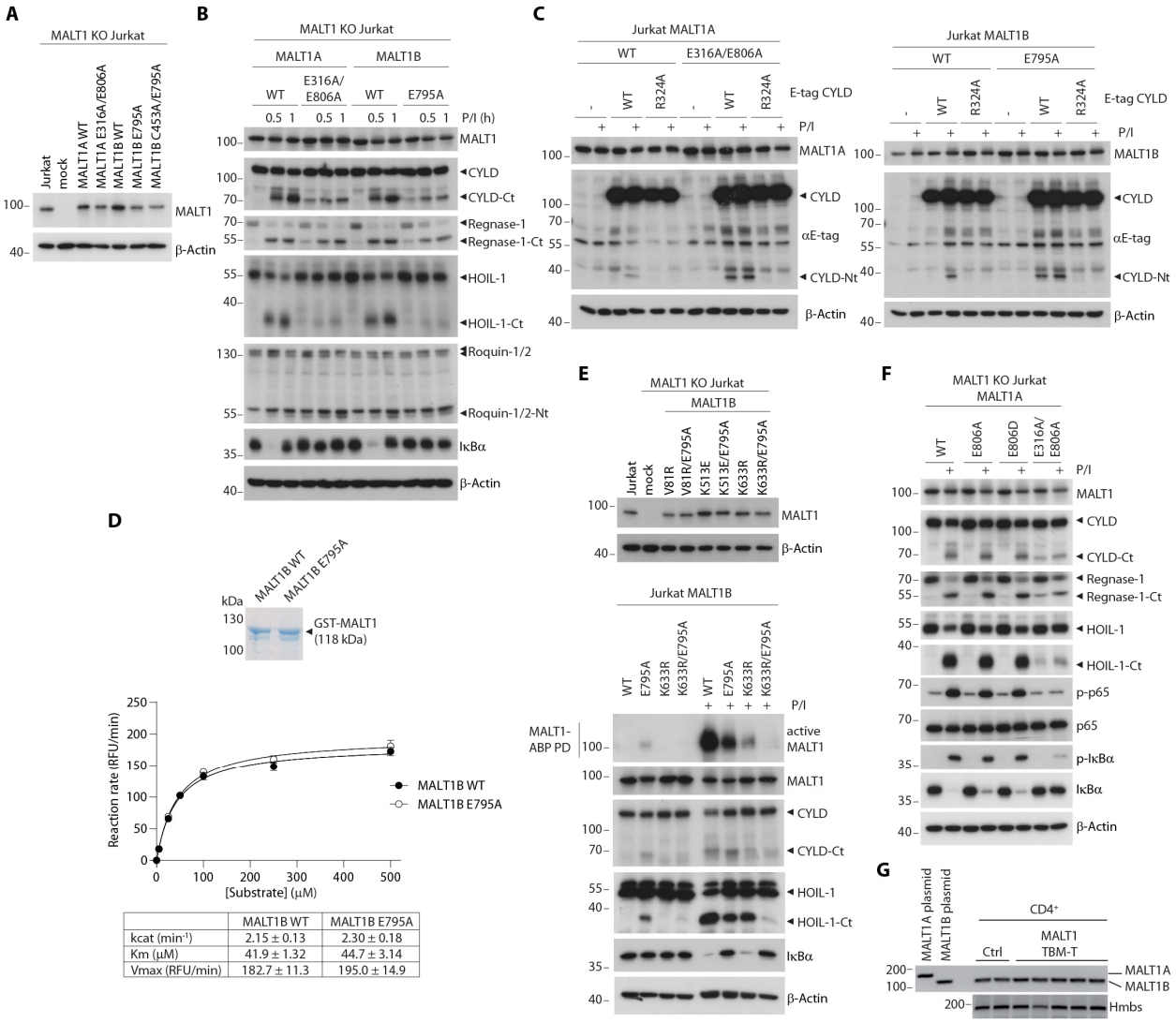


Fig. S5: Defective NF-κB and chronic MALT1 protease activation in *Malt1* TBM-T and *Traf6*-ΔT mice. (A and B) Analyses of NF-κB DNA binding (EMSA), canonical NF-κB signaling and MALT1 substrate cleavage (WB) after αCD3/CD28 stimulation of splenic CD4$^+$ T cells from control, *Malt1* TBM-T (A) and *Traf6*-ΔT (B) mice. Asterisks indicate unspecific signals. (C to F) Image stream analysis showing representative pictures (BF: bright field; scale bars: 10 μm) (C), histograms showing similarity scores of c-Rel and DAPI stain (D and E) and quantification of c-Rel and DAPI similarity scores from two independent experiments (F) in splenic CD4$^+$ T cells of control, *Malt1* TBM-T and *Traf6*-ΔT mice after P/I stimulation. (G) Flow cytometric analysis of IκBNS and ICOS positive CD4$^+$ T cells after 5 hours stimulation with αCD3/CD28 antibodies and co-treatment with the MALT1 inhibitor MLT-943 (1 μM). (H) Flow cytometric analysis of IκBNS and ICOS expression on CD4$^+$ T cells from spleen of control and *Malt1* TBM-T mice. All analyses were done with mice at 8-12 weeks of age. Littermate control mice were *Malt1*$^{fl/+}$*;CD4-Cre*$^-$, *Malt1*$^{fl/+}$;CD4-Cre$^+$, and *Malt1*$^{TBM/fl}$*;CD4-Cre*$^-$ or *Traf6*$^{fl/+}$*;CD4-Cre*$^+$*,Traf6*$^{fl/fl}$*;CD4-Cre*$^-$, and *Traf6*$^{fl/+}$*;CD4-Cre*$^-$ (black) for *Malt1* TBM-T (blue) or *Traf6*-ΔT (rose), respectively.



	MALT1B WT	MALT1B E795A
kcat (min⁻¹)	2.15 ± 0.13	2.30 ± 0.18
Km (μM)	41.9 ± 1.32	44.7 ± 3.14
Vmax (RFU/min)	182.7 ± 11.3	195.0 ± 14.9

Fig. S6: NF-κB activation and MALT1 protease activity upon loss of TRAF6-MALT1 interaction in Jurkat T cells. (A and B) Expression of MALT1 WT and mutant constructs (A) after reconstitution of MALT1 KO Jurkat T cells and analyses of MALT1 substrate cleavage and IκBα degradation (B) after reconstitution with MALT1A and MALT1B WT or T6BM mutants MALT1A E316A/E806A and MALT1B E795A untreated or stimulated with P/I by WB. (C) Transfection of E-tagged CYLD WT or the MALT1 cleavage insensitive mutant R324A in MALT1A WT or MALT1A E316A/E806A (left) and MALT1B WT or MALT1B E795A expressing (right) Jurkat T cells. Constitutive and P/I-inducible (60 min) cleavage of E-tagged CYLD was assessed by WB. (D) Coomassie gel of purified recombinant GST-MALT1B WT and GST-MALT1B E795A (upper panel) and measurement of Michaelis-Menten kinetics by increasing the concentration of Ac-LRSR-AMC substrate was used to calculate kcat, Km and Vmax. Graph and values depict the means ± SEM from 3 independent experiments. (E) Expression analyses of MALT1 WT and mutant constructs (upper panel) after lentiviral reconstitution of MALT1 KO Jurkat T cells and analyses of MALT1 activity by biotin-MALT1-ABP assay and substrate cleavage (lower panel) after reconstitution with MALT1B WT and mutants alone or in combination with the T6BM mutant (E795A) of untreated or P/I stimulated (60 min) cells by WB. (F) MALT1 KO Jurkat cells were reconstituted with patient-derived MALT1A E806D variant. Cells were left untreated or stimulated with P/I (30 min) and NF-κB signaling and MALT1 substrate cleavage was determined in WB. (G) Analysis of *Malt1A* and *Malt1B* mRNA expression by RT–PCR in $CD4^+$ T cells from $Malt1^{TBM/+}$ (control) and $Malt1^{TBM/TBM}$ mice. Migration of amplified *Malt1A* and *Malt1B* plasmid controls is shown and hydroxymethylbilane synthase (HMBS) mRNA served as internal control.

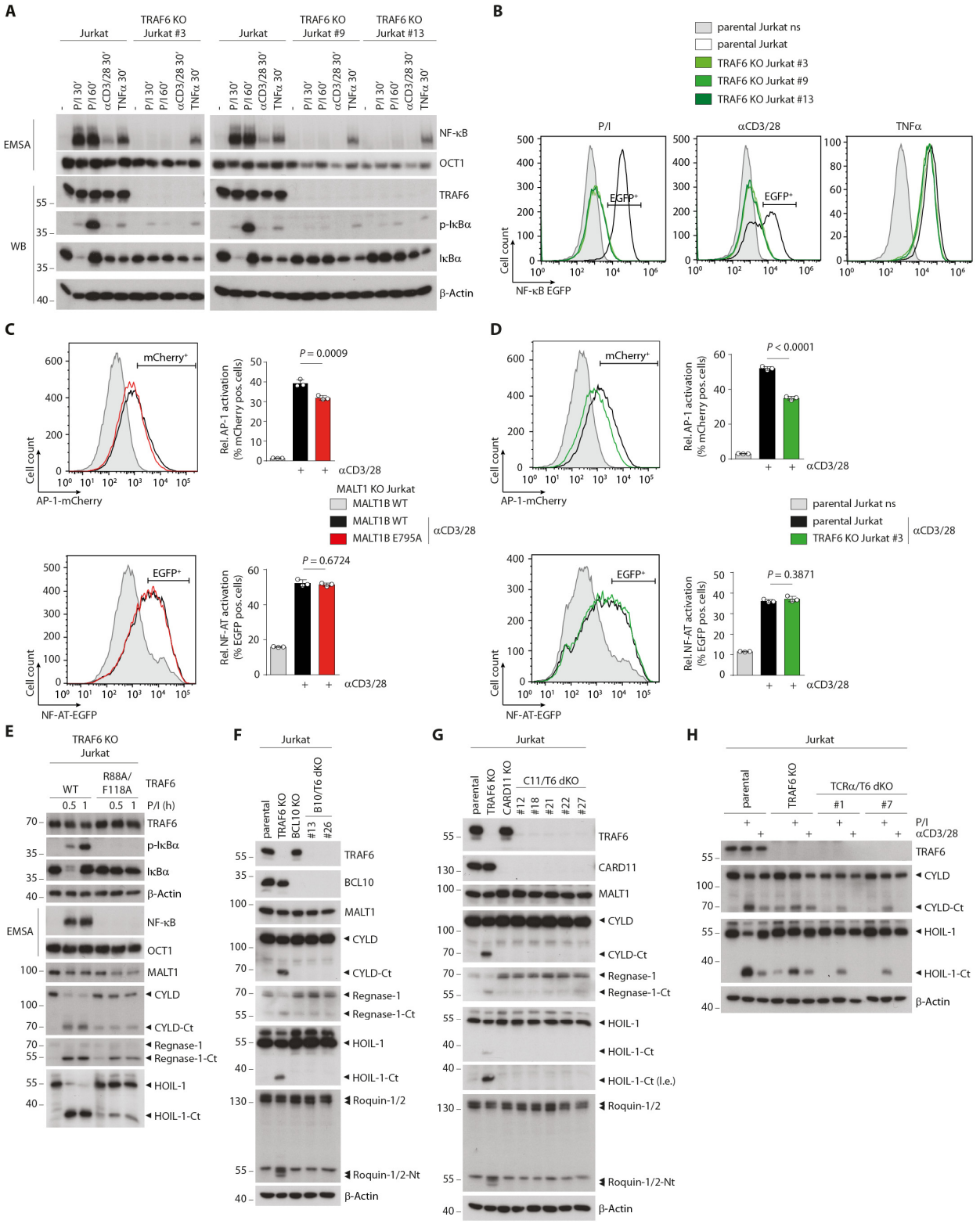


Fig. S7: NF-κB activation and MALT1 protease activity upon ablation of TRAF6 in Jurkat T cells. (A) Parental and TRAF6 KO Jurkat T cells were stimulated with P/I, αCD3/28 or TNFα. NF-κB activation was determined by WB and EMSA. (B) Parental and TRAF6 KO Jurkat T cells transduced with an NF-κB-EGFP reporter were stimulated with αCD3/28, P/I or TNFα and NF-κB activation was assessed by flow cytometry of EGFP positive cells. (C and D) MALT1B WT or E795A reconstituted Jurkat T cells (C) or TRAF6 KO Jurkat T cells (D) transduced with an AP-1-mCherry or NF-AT-EGFP reporter gene were

stimulated with αCD3/28 and NF-κB activation was assessed by flow cytometry of fluorescence positive cells. Bars show quantification of fluorescence positive cells from 3 independent experiments including the means ± SEM, and *P* values were calculated by ordinary one-way analysis of variance (ANOVA) combined with Dunnett's multiple comparisons. (E) TRAF6 KO Jurkat T cells reconstituted with TRAF6 WT or mutant R88A/F118A were analyzed for NF-κB signaling (WB and EMSA) and MALT1 substrate cleavage (WB) with and without P/I stimulation. (F and G) Parental, TRAF6 KO and different TRAF6/BCL10 (F) and TRAF6/CARD11 (G) dKO Jurkat T cells were analyzed for constitutive MALT1 substrate cleavage by WB. (H) Parental, TRAF6 KO and TRAF6/TCRα dKO Jurkat T cells were analyzed for constitutive and inducible MALT1 substrate cleavage after αCD3/CD28 or P/I stimulation (60 min) by WB.

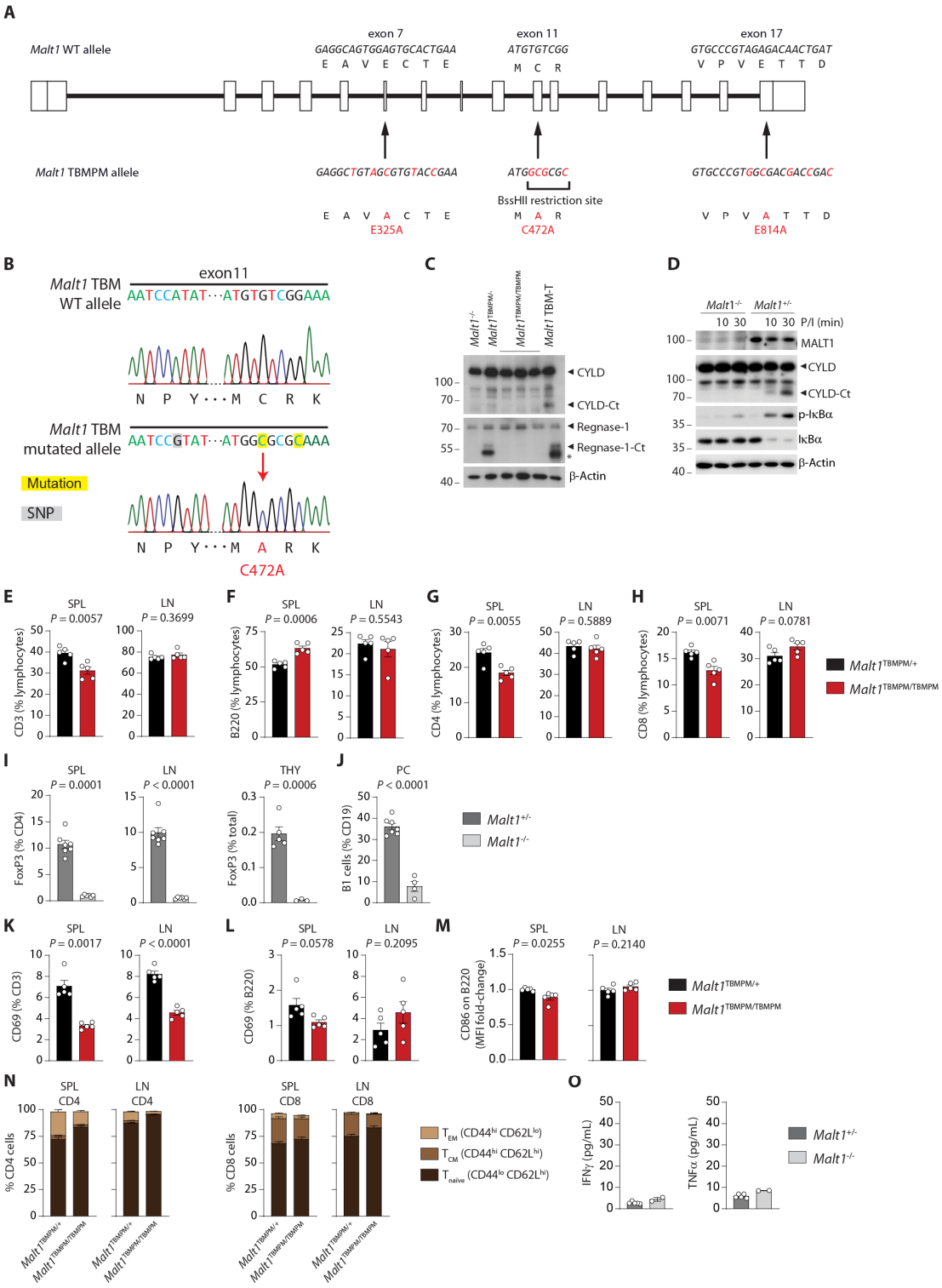


Fig. S8: Generation and analyses of *Malt1* TBMPM mice. (A) Schematic depiction of genomic organization of murine *Malt1* locus and sequences in exon 11 that were altered to mutate C472A (PM) in the *Malt1* TBM allele carrying the TBM1 (E325A) and TBM2 (E814A) mutations. (B) Sequencing of genomic region in exon 11 indicating paracaspase mutation C472A and the ES cell-derived silent SNP c.1323A/c.1323G serving as a marker for the *Malt1* TBM allele. (C) MALT1 substrate cleavage in isolated splenic CD4$^+$ T cells from *Malt1*$^{-/-}$, *Malt1*$^{TBMPM/+}$, *Malt1*$^{TBMPM/TBMPM}$ and *Malt1*$^{TBM/fl}$;CD4$^-$Cre$^+$ (*Malt1* TBM-T) mice. (D) IκBα phosphorylation and degradation and CYLD cleavage in splenic CD4$^+$ T cells

isolated from *Malt1*$^{-/-}$ and *Malt1*$^{+/+}$ mice. (E to H) Relative numbers of CD3+ (E) T cells, B220^{+} (F) B cells, CD4^{+} (G) T cells and CD8^{+} (H) T cells from spleen (SPL) and lymph nodes (LN) of *Malt1*$^{TBMPM/+}$ and *Malt1*$^{TBMPM/TBMPM}$ mice. (I and J) Relative numbers of CD4^{+}Foxp3^{+} Treg cells from spleen, lymph nodes and thymus (I) and B1 cells from peritoneal cavity (J) of *Malt1*$^{+/-}$ and *Malt1*$^{+/+}$ mice. (K and L) Relative numbers of CD3^{+}CD69^{+} T (K) or B220^{+}CD69^{+} (L) B cells from spleen and lymph nodes of *Malt1*$^{TBMPM/+}$ and *Malt1*$^{TBMPM/TBMPM}$ mice. (M) Fold change of CD86 MFI on B220^{+} B cells from spleen and lymph nodes between *Malt1*$^{TBMPM/+}$ and *Malt1*$^{TBMPM/TBMPM}$ mice. (N) Relative numbers of $T_{naïve}$, T_{CM} and T_{EM} CD4^{+} and CD8^{+} T cells from spleen and lymph nodes of *Malt1*$^{TBMPM/+}$ and *Malt1*$^{TBMPM/TBMPM}$ mice. (O) Concentrations of IFNγ and TNFα in sera of *Malt1*$^{+/-}$ and *Malt1*$^{-/-}$ mice. All analyses were performed with animals 8 weeks after birth. Each dot represents 1 mouse (E-N: 3 to 7 mice per group). All bars show the means ± SEM, and *P* values were calculated by unpaired *t* test with Welch's correction.

Table S1: CRISPR guides and homology templates.

Description	Name	DNA sequence
CRISPR-Cas9 targeting of *Malt1* (murine)	T6BM1 sgRNA	5'-GAGGCAGTGGAGTGCACTGA-3'
	T6BM2 sgRNA	5'-ATTTCATCAGTTGTCTCTAC-3'
	PM sgRNA	5'-TGTTCCTGTTGGATATGTGT-3'
	T6BM1 ssODN HDR	5'-TCTCTGTTGACTTAATGACTACCCTTCCTAAGA TATGAGTTACAATATGTTCACATTTGTTTTCTCTA AATAAGGAAGAACAGATGAGGCTGTAGCGTGTA CCGAGGTAGTGCAATCCTTTGGTTCAAGACCAAG AGTCCTCATGCTGCATGCTGGTTACTCTGGGGAG AGGTGGAGGTCCTAGCTCACT-3'
	T6BM2 ssODN HDR	5'-CCAGACAGGTGTCATTGCAGCCGGACTCCACA CACATTCATTTCAAATTATCCCCCCCACCACTACT GCCAGTTTGGTAGATCCAATGTGCCCGTGGCGA CGACCGACGAAATGCCATTCAGTTTTTCTGACAG GCTTATGATTTCTGAAAACTGACCTTCATGGTTTT GAAAATTAGAATAGTTACAGTAATCT-3'
	PM ssODN HDR	5'-CCTGTTGATGCTCCAAATCCATATAGGTCTGA AAATTGCCTATGCGTACAAAACATACTGAAATTA ATGCAAGAAAAGGAGACTGGCCTGAATGTGTTC CTGTTGGATATGGCGCGCAAAAGGTAAAATGTC TCATCTCTCTATCAAGTAGCAACCTTGACAAAGT CTATGTAAGGCAATTCTGTACGGTGGTAA-3'
CRISPR-Cas9 targeting of *Traf6* (Jurkat)	Traf6 exon 1 sgRNA	5'-TGTTACAGCGCTACAGGAGC-3'
	Traf6 exon 2 sgRNA	5'-ATGGTGAAATGTCCAAATGA-3'
CRISPR-Cas9 targeting of *TCR*α (Jurkat)	TCRα exon 1 sgRNA	5'-ACAAAACTGTGCTAGACATG-3'

Table S2: Genotyping primers.

Description	Name	DNA sequence
Malt1 TBM1 genotyping	T6BM1 fwd general	5'-GCAAAGAAACCCTAAACATC-3'
	T6BM1 rev WT-specific	5'-CCTTCAGTGCACTCCACT-3'
	T6BM1 rev Mut-specific	5'-CCTTCGGTACACGCTACA-3'
Malt1 TBM2 genotyping	T6BM2 fwd general	5'-ACGGATAAGAGTGGAGTGAT-3'
	T6BM2 rev WT-specific	5'-ATTTCATCAGTTGTCTCT-3'
	T6BM2 rev Mut-specific	5'-ATTTCGTCGGTCGTCGCC-3'
Malt1 knockout genotyping	Malt1 flox del fwd	5'-CTAGTCAGTCACCAGCTCAG-3'
	Malt1 flox del rev	5'-CTGGCTAACCAATCCTCAAAAC-3'
	Malt1 flox rev	5'-CAGTTCTCAATGCCAACGCAC-3'
FoxP3-Cre genotyping	FoxP3-Cre TG fwd	5'-CGGGTCAGAAAGAATGGTGT-3'
	FoxP3-Cre TG rev	5'-CAGTTTCAGTCCCCATCCTC-3'
	FoxP3-Cre ctrl fwd	5'-CAAATGTTGCTTGTCTGGTG-3'
	FoxP3-Cre ctrl rev	5'-GTCAGTCGAGTGCACAGTTT-3'
CD4-Cre genotyping	CD4-Cre fwd	5'-ACCAGCCAGCTATCAACTCG-3'
	CD4-Cre rev	5'-TTACATTGGTCCAGCCACC-3'
Traf6 genotyping	Traf6 fwd	5'-CATGGCTTGTTACCTCTGCTC-3'
	Traf6 wt/flox rev	5'-TCCAGCAGTATTTCATTGTCAAC-3'