Human immune disorder associated with homozygous hypomorphic mutation affecting MALT1B splice variant

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T and B cell antigen receptor (TCR/BCR) ligation on lymphocytes triggers activation of canonical NF-κB signaling, which is critical for mounting an adaptive immune response. The CARD11/CARMA1-BCL10-MALT1 (CBM) signaling complex acts as the gatekeeper for antigen receptor-induced NF-κB activation. In activated T cells, MALT1 serves a dual role as a scaffold and a protease. While recruitment of the ubiquitin ligase TRAF6 to MALT1 triggers NF-κB signaling, the MALT1 protease cleaves distinct substrates to modulate T cell effector functions. Germline mutations in all CBM components have been associated with a phenotypically diverse set of human primary immunodeficiencies termed 'CBM-opathies'[1]. To date, nine patients have been identified comprising six different mutations in the *MALT1* gene that are linked to combined immune deficiency (CID)[1,2]. Importantly, all known mutations cause loss-of-function due to absence or strong reduction of MALT1 protein expression. Here we describe the first case of a severe immune syndrome caused by a homozygous hypomorphic mutation in the human *MALT1* gene.

A 19-year-old Turkish girl of parents who are first-degree cousins presented with a history of recurrent chronic purulent otitis and secondary hearing-poorness at admission. She was treated and hospitalized several times for bronchopneumonia. Physical examination

diagnosed a widespread seborrheic dermatitis on the scalp, psoriasis on the hands and feet extensor regions, severe warts on the hands and bilateral axillary and cervical lymphadenomegaly. High-resolution chest tomography revealed superior segmental linear atelectasis with bronchiectasis in the right lung lower lobe. In axillary and retropectoral regions 2.7x12mm conglomerate lymph nodes were determined. In abdominal ultrasonography, splenomegaly and grade 1 hydroureteronephrosis in the right kidney as well as multiple lymphadenopathies in the epigastric and para-aortic regions were found. An excisional biopsy revealed follicular hyperplasia of an enlarged lymph node, but malignancy was excluded. Scalp biopsies showed hyperplasia in squamous epithelium and psoriasis with perivascular lymphocyte infiltration in the dermis, diagnosed as psoriasis with cutaneous symptoms.

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Since clinical features indicated a primary immune deficiency with additional signs of autoimmunity, targeted next-generation sequencing was performed using a primary immune deficiency research panel comprising 264 genes. We identified a heterozygous missense mutation c.2080A>G (p.Met694Val;M694V) in the *MEFV* gene, an autosomal recessive

mutation associated with familial Mediterranean fever (FMF). Clinical pathology of FMF with recurrent fever episodes is very distinct from what is observed in the patient, but some contribution cannot be completely excluded[3]. A single homozygous missense mutation c.2418G>C (p.Glu806Asp;E806D) in the *MALT1* gene was identified (**Figure E2**). Sanger sequencing verified the homozygous *MALT1* c.2418G>C mutation in exon 17 of the patient, while the parents, who have no clinical symptoms, were heterozygous for the mutation (**Figure 1A**). With *MALT1A* and *MALT1B* (Δexon 7) two splice variants exist and transcripts for both isoforms are expressed in patient and control PBMCs (**Figure 1B and E3**). Thus, the *MALT1* c.2418G>C missense mutation leads to Glu806Asp (E806D) and Glu795Asp (E795D) substitution in MALT1A and MALT1B protein, respectively (**Figure 1C**)[4]. In contrast to all other known human MALT1 loss-of-function mutations, the MALT1 E806D/E795D mutant protein is expressed at WT levels in patient PBMCs (**Figure 1D**). In line with this, MALT1B WT and E795D are expressed at equivalent levels upon stable viral transduction in MALT1 KO Jurkat T cells, while the previously identified MALT1B W569S mutant (corresponding to MALT1A W580S) is unstable, but can be stabilized by an allosteric MALT1 inhibitor (**Figure E4A-C**)[5,6].

The *MALT1* c.2418G>C exchange occurs directly within the C-terminal evolutionarily conserved TRAF6 binding motif 2 (T6BM2) of MALT1 (**Figure 1C and E5**). Recruitment of TRAF6 via T6BMs to MALT1 is essential for NF-κB activation, but is not directly connected to MALT1 protease activation in T cells[4]. While MALT1A contains two functional T6BMs (T6BM1 and T6BM2), MALT1B lacks T6BM1 encoded by the alternatively spliced exon 7 and thus encodes only the T6BM2, which is mutated in the patient (**Figure 1C**). Indeed, just like MALT1 T6BMs destruction by E/A replacement[4], the MALT1B E795D or MALT1A E806D patient mutation abolishes or diminishes TRAF6 binding, respectively. Stronger

TRAF6 interaction of MALT1A is only abrogated upon combined mutation of T6BM1 and T6BM2 (**Figure E6**).

In the context of MALT1A, we now mutated the C-terminal T6BM2 in combination with the T6BM1, to show that both sites are required for full TRAF6 recruitment (**Figure E6**)

To test whether the Glu/Asp replacement generates a hypomorphic variant that primarily affects activity of the shorter MALT1B isoform, we introduced Strep-tag II (ST)-tagged MALT1A WT, MALT1A E806D, MALT1B WT and MALT1B E795D into MALT1 KO Jurkat T cells containing an NF-κB-dependent EGFP reporter. Stable transduction led to expression of all MALT1 variants at levels equivalent to endogenous MALT1 in parental Jurkat T cells (**Figure 1E and F; E7A and B**). NF-κB activation was analyzed by detecting EGFP-positive cells using flow cytometry after PMA/Ionomycin (P/I) or αCD3/CD28 antibodies to mimic TCR/CD28 co-stimulation and the cytokine TNFα (**Figure E7C and D**). As expected, TNFα-triggered NF-κB activation was not affected by MALT1 loss or mutations (**Figure 1G and H**). Whereas the E806D mutation in MALT1A did not decrease NF-κB activation after P/I or TCR/CD28 co-stimulation, the identical E795D mutation in MALT1B abolished NF-κB activation in response to T cell activation. Human blood CD4+ T cells express ~5-fold higher levels of MALT1B over MALT1A[4]. To mimic such a scenario *in vitro*, we co-transduced MALT1 KO Jurkat T cells with untagged MALTB E795D and increasing amounts of ST-tagged MALT1A WT or E806D (**Figure 1I**). Indeed, low expression of MALT1A WT or E806D was unable to rescue defective TCR/CD28- and P/I-induced NF-κB activation in the presence of MALT1B E795D. MALT1A E806D was even more severely compromised in rescuing NF-κB signaling, demonstrating that the relative expression of MALT1A E806D and MALT1B E795D in patient T cells strongly influences the severity of the hypomorphic *MALT1* c.2418G>C loss-of-function mutation.

We describe a patient-derived homozygous *MALT1* c.2418G>C transversion, which leads to a unique hypomorphic MALT1 alteration that selectively disrupts signaling of the MALT1B isoform (**Figure 1J**). The immune pathology is manifested by symptoms of immune deficiency such as chronic ear and bronchial infections and at the same time autoimmunity as evident from skin psoriasis, lymphadenopathies and autoantibodies. We provide evidence that the complex immune disorder may be linked to the relative expression of MALT1A and MALT1B in distinct immune cells. Defective activation in primarily MALT1B E795D-expressing T cells may explain compromised patient immune responses and immune deficiencies. How the *MALT1* c.2418G>C mutation is associated with autoimmunity needs further investigations. Interestingly, psoriasis is driven by CARD14-MALT1 signaling in keratinocytes[7] and we speculate that imbalanced MALT1A/MALT1B signaling in certain immune or non-immune cells may result in overshooting immune or inflammatory responses causing autoimmunity.

Currently, the patient continues to suffer from recurrent ear infections despite prophylactic treatment with intravenous immunoglobulin replacement therapy, antibiotic prophylaxis and mycophenolate mofetil for preventing and treating autoimmunity. The patient is enrolled for hematopoietic stem cell therapy, but she is currently quarantined in her home in Eastern Turkey due to the COVID-19 pandemic.

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Table 1: Laboratory parameters and lymphocytes.

	Patient	Normal range
Serum Immunoglobulins (mg/dl)[1]		
IgA	<5	46.5-221
IgM	236	75.0-198.5
IgG	2930	830-1820
IgG1	2520	528-1384
IgG2	<37.6	147-610
IgG3	296	21-152
IgG4	<5.7	15-202
Antibody responses		
Anti-Tetanus IgG (IU/ml)	0.11	normal
Anti-H. influenza B (µg/ml)	1.05	normal
Complement (mg/dl)		
C3	79	90-180
C4	6	10-40
Autoantibodies		
ANA (titer)	1/320 (+)	increased
C-ANCA (titer)	1/64 (+)	increased
anti-MPO (RU/ml)	3	<20
Anti-PR3 (RU/ml)	38	<20
Direct combs test	++	negative
Lymphocytes (%)[2]		
CD3+	74	67.8-76.3
CD3+CD4+	42	58.7-70.6
CD3+CD8+	50	24.8-34.6
CD19+	6	8.8-13.1

[1] Healthy Turkish children >16 y[8]
[2] Healthy European/Caucasian females >20 y[9]

FIGURE LEGEND

Figure 1: A) Sanger sequencing identified heterozygous and homozygous c.2418G>C mutation in the parents (upper panel) and patient (lower panel). B) Scheme of the *MALT1* gene depicts alternatively spliced Exon 7 in *MALT1A* and *MALT1B* and c.2418G>C mutation in Exon17. C) Structure of MALT1A and MALT1B (ΔExon7) protein including the TRAF6 binding motifs (T6BM) and the E806D or E795D mutation in T6BM2. D) Western blot for MALT1 expression in blood PBMCs of control and patient. E and F) Expression of ST-tagged MALT1A and MALT1B WT and mutant proteins was assessed by Western blot. Asterisk marks unspecific band. G and H) Quantification of NF-κB activation by EGFP-positive Jurkat T cells in MALT1A (G) or MALT1B (H) WT and mutant expressing cells after P/I, αCD3/CD28 and TNFα stimulation. I) Quantification of NF-κB activation by EGFP-positive Jurkat T cells transduced with untagged MALT1B E795D and increasing amounts of ST-tagged MALT1A WT or E806D after P/I (upper panel) or αCD3/CD28 (lower panel). Western blot of MALT1B E795D and MALT1A WT or E806D in co-transduced cells. Data represent means ± SEM of three (G and H), four (I: αCD3/CD28) or five (I: P/I) biological replicates. ns: not significant, ****p $\leq$ 0.0001, ***p $\leq$ 0.001, ** p $\leq$ 0.01, * p $\leq$ 0.05. J) Mechanistic model of the differential effects of the MALT1A E806D and the MALT1B E795D mutation on NF-κB signaling in T cells.

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Table 1: Laboratory parameters and lymphocytes.

	Patient	Normal range
Serum Immunoglobulins (mg/dl)[1]		
IgA	<5	46.5-221
IgM	236	75.0-198.5
IgG	2930	830-1820
IgG1	2520	528-1384
IgG2	<37.6	147-610
IgG3	296	21-152
IgG4	<5.7	15-202
Antibody responses		
Anti-Tetanus IgG (IU/ml)	0.11	normal
Anti-H. influenza B (µg/ml)	1.05	normal
Complement (mg/dl)		
C3	79	90-180
C4	6	10-40
Autoantibodies		
ANA (titer)	1/320 (+)	increased
C-ANCA (titer)	1/64 (+)	increased
anti-MPO (RU/ml)	3	<20
Anti-PR3 (RU/ml)	38	<20
Direct combs test	++	negative
Lymphocytes (%)[2]		
CD3+	74	67.8-76.3
CD3+CD4+	42	58.7-70.6
CD3+CD8+	50	24.8-34.6
CD19+	6	8.8-13.1

[1] Healthy Turkish children >16 y[8]
[2] Healthy European/Caucasian females >20 y[9]

FIGURE LEGEND

Figure 1: A) Sanger sequencing identified heterozygous and homozygous c.2418G>C mutation in the parents (upper panel) and patient (lower panel). B) Scheme of the *MALT1* gene depicts alternatively spliced Exon 7 in *MALT1A* and *MALT1B* and c.2418G>C mutation in Exon17. C) Structure of MALT1A and MALT1B (ΔExon7) protein including the TRAF6 binding motifs (T6BM) and the E806D or E795D mutation in T6BM2. D) Western blot for MALT1 expression in blood PBMCs of control and patient. E and F) Expression of ST-tagged MALT1A and MALT1B WT and mutant proteins was assessed by Western blot. Asterisk marks unspecific band. G and H) Quantification of NF-κB activation by EGFP-positive Jurkat T cells in MALT1A (G) or MALT1B (H) WT and mutant expressing cells after P/I, αCD3/CD28 and TNFα stimulation. I) Quantification of NF-κB activation by EGFP-positive Jurkat T cells transduced with untagged MALT1B E795D and increasing amounts of ST-tagged MALT1A WT or E806D after P/I (upper panel) or αCD3/CD28 (lower panel). Western blot of MALT1B E795D and MALT1A WT or E806D in co-transduced cells. Data represent means ± SEM of three (G and H), four (I: αCD3/CD28) or five (I: P/I) biological replicates. ns: not significant, ****$p \leq 0.0001$, ***$p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$. J) Mechanistic model of the differential effects of the MALT1A E806D and the MALT1B E795D mutation on NF-κB signaling in T cells.

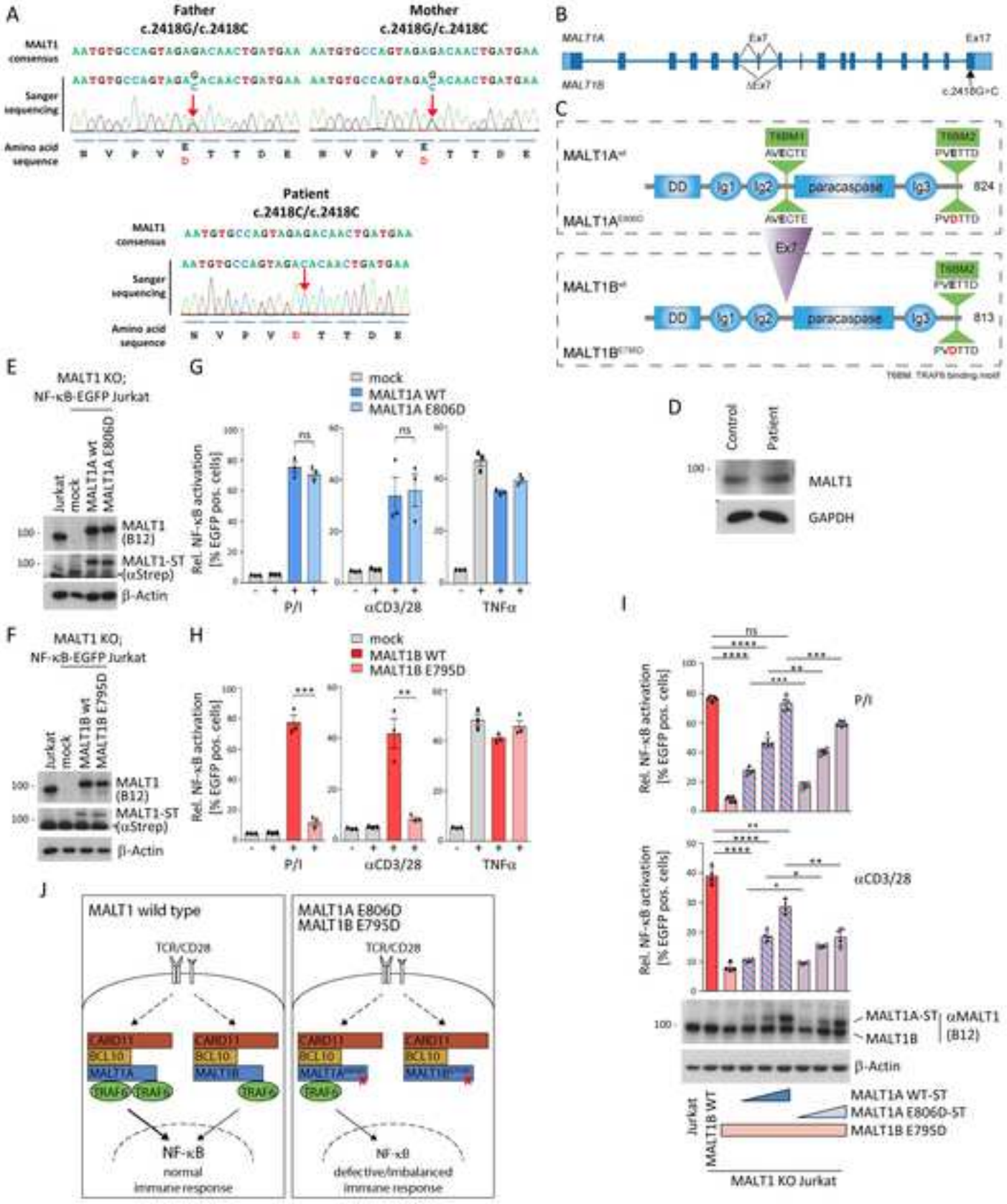

Figure 1

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METHODS

Informed consent was obtained from the patient and her parents.

Genomic exome library construction and targeted next generation sequencing

Targeted next-generation sequencing (TNGS) was performed using an Ion S5™ Sequencer. The Ion AmpliSeq™ Primary Immune Deficiency (PID) Research Panel comprising 264 genes was used and analysis of mutations within 264 PID genes was done with Ion reporter software. The potential functional impacts of the identified variants were assessed using VarSome.

Purification of peripheral blood mononuclear cells (PBMC)

To purify PBMCs for flow cytometry and Western blot analysis, blood was drawn from donors into Lithium-Heparin tubes and centrifuged (300 x g without brake, 10 min, RT). Plasma (upper layer) was removed and the buffy coat containing MNCs and platelets (intermediate layer) was transferred and mixed with PBS to a total volume of 35 mL. Diluted sample was added carefully on top of 15 mL of Lymphoprep density gradient medium (StemCell Technologies, Inc.) and centrifuged (160 x g without brake, 20 min, RT) to purify MNCs. Approximately 20 mL of supernatant containing platelets was removed and remaining sample was centrifuged (350 x g without brake, 20 min, RT). Cells from the intermediate layer (MNCs) were separated, washed twice in 10 mL Buffer 1 (PBS containing 0.1% BSA, 2 mM EDTA (pH 7.4), sterile filtered) (300 x g, 8 min, 4°C), and resuspended in medium (RPMI, 10% FCS, 1% Pen/Strep, 50 µM β-Mercaptoethanol). Protein expression in PBMCs was determined by Western Blot using anti-MALT1 (B-12, Santa Cruz) and anti-GAPDH-HRP (14C10, Cell Signaling) antibodies.

Immunologic phenotyping by flow cytometry

Lymphocyte populations were determined from single-cell PBMC suspensions stained with anti-CD3-APC (HIT31, 1:200, Biolegend), anti-CD19-PEDazzle (SJ25C1, 1:200, Biolegend), anti-CD4-FITC (RPA-T4, 1:200, Biolegend), and anti-CD8-PECy5 (RPA-T8, 1:200, Biolegend). To investigate lymphocyte activation, anti-CD69-PECy5 (FN50, 1:200, Biolegend) was used. $2x10^5$ PBMCs were washed twice with PBS and stained with Fixable Viability Dye eFluor 780 (1:1000) for 30 min at 4°C to distinguish live from dead cells. Cells were washed with FACS buffer (3% FCS in PBS) and treated with anti-CD16/CD32 (Fc block) for 20 min at RT. Cells were stained with fluorescent antibodies for 20 min at RT, washed with FACS buffer, and analyzed on an Attune Acoustic Focusing Cytometer or on an BD Fortessa Cytometer.

mRNA extraction and reverse transcription (RT)-PCR

mRNA was isolated (Qiagen RNeasy Kit) from $5x10^6$ PBMCs and 400 ng of RNA was used for reverse transcription (Verso cDNA synthesis kit, Thermo Fisher). RT-PCR was performed using LongAmp® Taq DNA Polymerase (NEB) with primers in flanking exons (ex6 primer: ACCGAGACAGTCAAGATAGC and ex9/10 primer: GACTTTGTCCTTTGCCAAAGG) detecting both isoforms MALT1A (146 bp) and MALT1B (113 bp). 30 ng cDNA or MALT1A/MALT1B plasmid DNA was used as template. GAPDH/RPII served as a control. PCR products were loaded on a 2-3 % agarose gel and visualized with UV light using INTAS gel documentation system.

HEK293 transfection and Strep-PD

HEK293 cells were grown in DMEM (10% FCS, and 100 U/ml penicillin/ streptomycin) and transfection of HA-TRAF6 (pEF vector) and MALT1-FlagStrepII constructs (pHAGE vector) using calcium phosphate precipitation. Cells were lysed in co-immunoprecipitations (co-IP)

buffer (25 mM HEPES (pH 7.5), 150 mM NaCl, 0.2% NP-40, 10% glycerol, 1 mM DTT, 10mM sodium fluoride, 8 mM β-glycerophosphate, 300 µM sodium vanadate and Roche protease inhibitor cocktail), subjected to Strep-PD using Strep-Tactin sepharose beads and TRAF6-MALT1 binding was analyzed by Western Blot.

Lentiviral transduction of Jurkat T cells

Jurkat T cell lines were grown in RPMI medium supplemented with 10 % FCS and 100 U/ml P/S. To generate stable MALT1-expressing cell lines, MALT1 KO;NF-κB-EGFP Jurkat T cells[E1] were lentivirally transduced with constructs in which MALT1A, MALT1A E806D, MALT1B, MALT1B E795D were cloned into pHAGE-hΔCD2-T2A backbone vector. For this, lentiviruses were produced in HEK293T cells. In brief, 2x 10^6 HEK293T cells were seeded in a 10 cm^2 dish in 8 ml DMEM medium one day prior to transfection. The following day, HEK293T cells were transfected with 1 µg of the lentiviral envelope plasmid pMD2.G (Addgene #12259; gift D. Trono), 1.5 µg of the packaging vector psPAX2 (Addgene #12260; gift D. Trono), and 2 µg MALT1 transfer plasmid (pHAGE-hΔCD2-T2A-MALT1-StrepTagII) using X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics) according to the manufacturer's instructions. After 3 days, the supernatant containing the virus particles was removed and sterile filtered (0.45 µM) before adding virus with 8 µg/ml polybrene to 5x10^5 MALT1-deficient Jurkat T cells. After 24 h cells were washed with PBS (three times) and re-suspended in 1 ml fresh RPMI medium. After two weeks, transduction efficiency was assessed by analyzing surface expression of hΔCD2 in flow cytometry with anti-CD2-APC (RPA-2.10, 1:400, invitrogen). Western Blots after transfection or transduction were carried out using anti-MALT1 (B-12; Santa Cruz), anti-β-Actin (C4, Santa Cruz), anti-HA (12CA5) and anti-StrepMAP-HRP (IBA).

NF-κB-EGFP reporter analyses in Jurkat T cells

MALT1 KO;NF-κB reporter Jurkat T cells stably transduced with mock or different MALT1 constructs were stimulated with PMA (200 ng/ml)/Ionomycin (300 ng/ml) (P/I) (Calbiochem), αCD3 (0.3 µg)/αCD28 (1 µg) antibodies in presence of anti-murine IgG1 (0.5 µg) and IgG2a (0.5 µg) (all BD Pharmingen), or TNFα (20 ng/ml, Biomol) for 5h. EGFP expression was determined by flow cytometry and quantification was done by gating on EGFP-positive cells.

References

E1 Gehring T, Erdmann T, Rahm M, Graß C, Flatley A, O'Neill TJ, et al. MALT1 Phosphorylation Controls Activation of T Lymphocytes and Survival of ABC-DLBCL Tumor Cells. Cell Reports. 2019;29(4):873-88.e10.

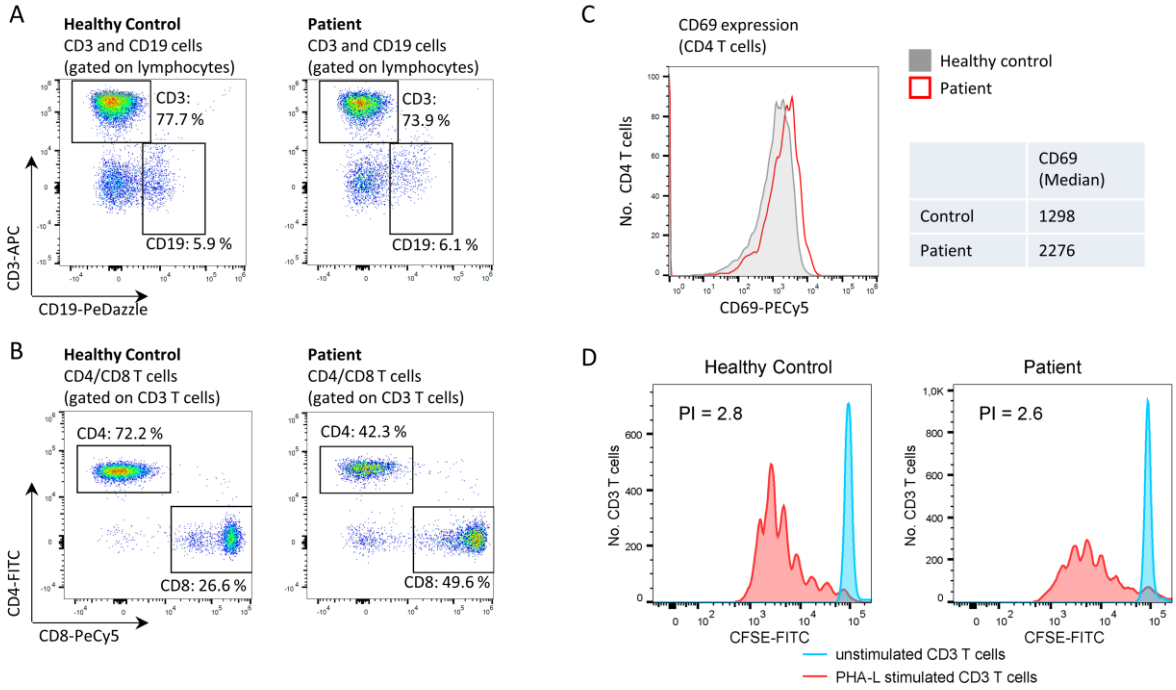

Figure E1: A) FACS of CD3+ T cells and CD19+ B cells in blood PBMCs from female patient and healthy control. B) FACS analyses of CD4+/CD8+ T cell ratio in peripheral CD3+ T cells from blood of patient and control. C) Histogram comparing expression of activation marker CD69 on blood CD4+ T cells in healthy control and patient. D) Histogram graphics of unstimulated and PHA-L stimulated CD3+ T cells (gated on CD3-APC) representing intact *in vitro* T lymphocyte proliferation response by CFSE-FITC labeling method of healthy control and patient. Proliferation Index (PI) is depicted.

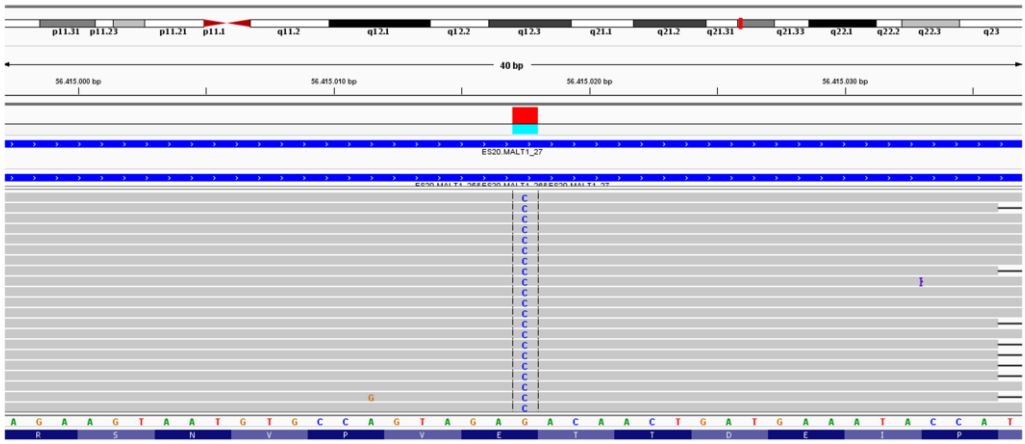

Figure E2: Targeted next generation sequencing (TNGS) analysis result of the patient showing homozygous c.2418G>C missense mutation in *MALT1* gene leading to Glu806Asp (E806D) exchange in MALT1A.

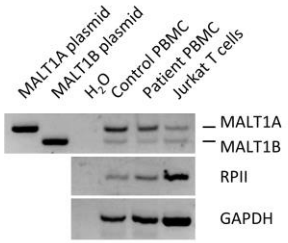

Figure E3: Analysis of MALT1 isoform expression by RT-PCR in PBMCs isolated from the blood of the patient and a healthy control using ex6-ex9/10 primers amplifying both isoforms. Jurkat T cells as well as MALT1A or MALT1B plasmid DNA served as controls. Fragments for MALT1A (146bp) and MALT1B (113bp) are indictated. GAPDH and RPII amplification were used as controls.

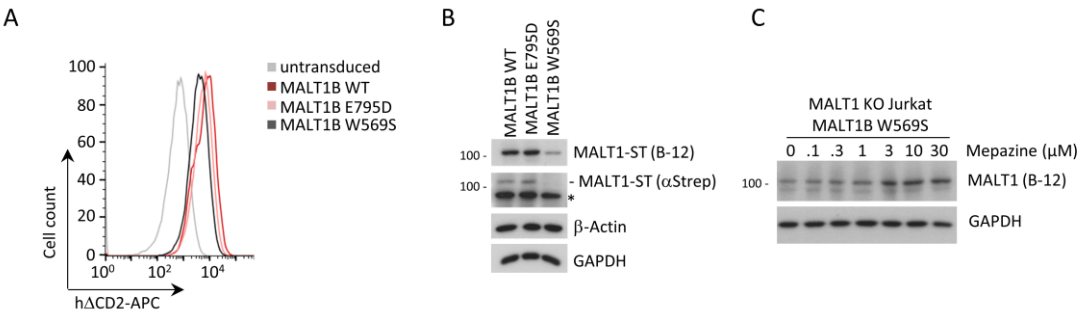

Figure E4: A) Transduction efficiency of MALT1 KO;NF-κB-EGFP Jurkat T cells with ST-tagged MALT1 WT, E795D and W569S mutants was monitored by co-expression of the surface marker hΔCD2 in FACS. B) Protein expression of MALT1B WT and mutant proteins were assessed by Western blot using MALT1 αStrep antibodies. Asterisk marks unspecific band. C) Jurkat T cells expressing MALT1B W569S were incubated for 5h with increasing concentrations of allosteric MALT1 inhibitor S-Mepazine and MALT1 protein stabilization was assessed by Western blotting.

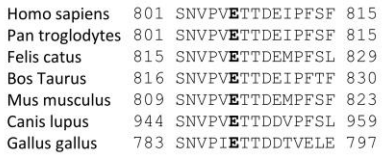

Figure E5: Evolutionary conservation of MALT1A E806 among different species.

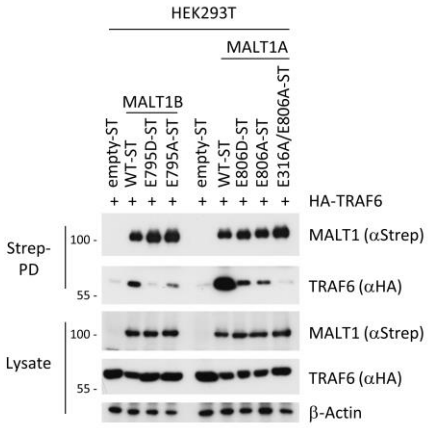

Figure E6: HEK293 cells were transfected with HA-TRAF6 and MALT1-FlagStrepII WT and mutant constructs. Strep-PD was performed and MALT1-ST and HA-TRAF6 binding was analyzed by Western blot.

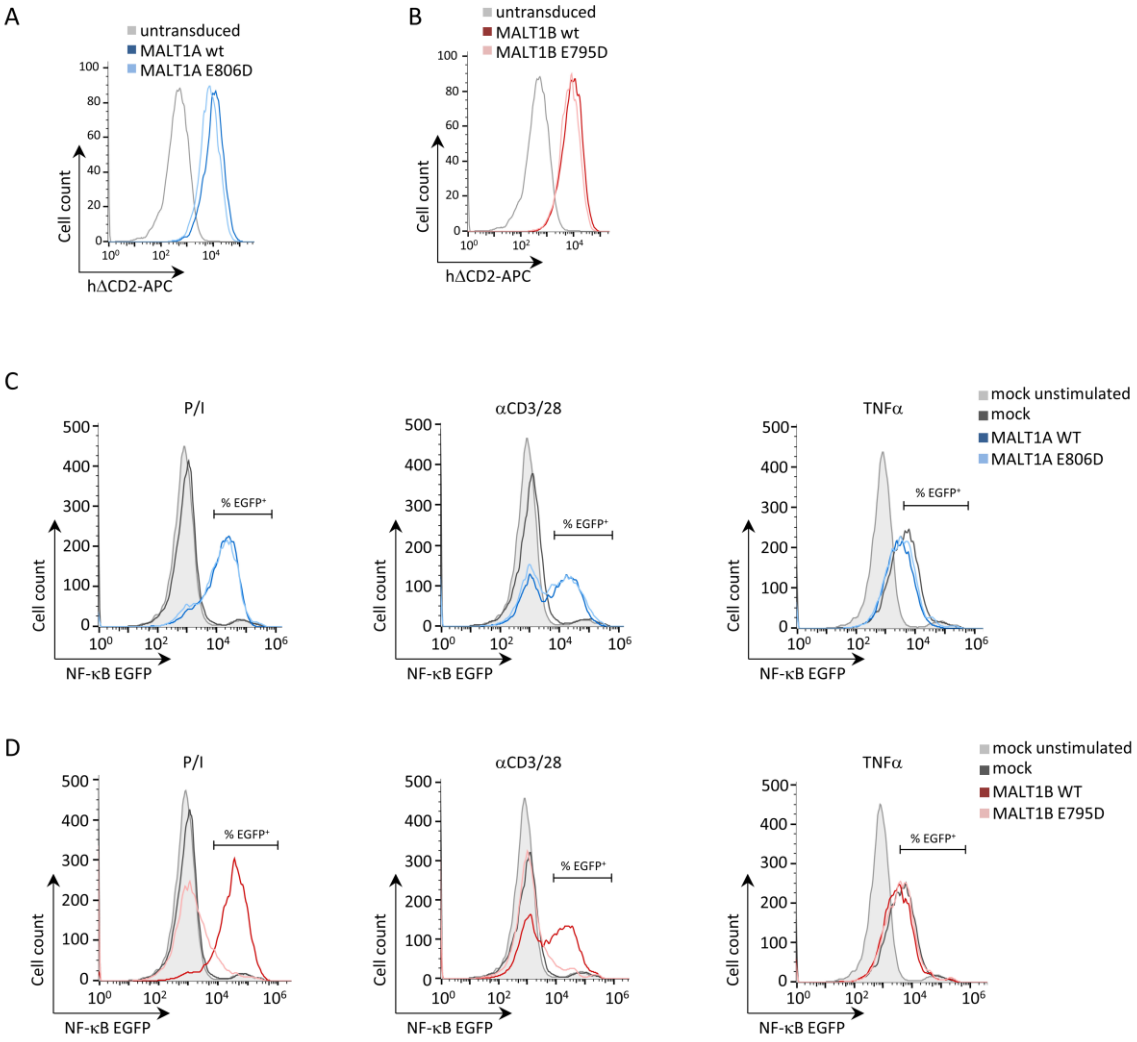

Figure E7: A and B) Transduction efficiency of MALT1 KO;NF-κB-EGFP Jurkat T cells with MALT1A (A) and MALTB (B) WT and mutant constructs was monitored by co-expression of surface marker hΔCD2 in FACS. C and D) Induction of the NF-κB-EGFP reporter in MALT1 KO Jurkat T cells after transduction of mock, MALT1A WT, MALT1A E806D, MALT1B WT and MALT1B E795D as determined by FACS after P/I, αCD3/CD28, or TNFα stimulation. Gate depicts the EGFP-positive cells used for quantification in Figure 1G and H.