TARP is an immunotherapeutic target in acute myeloid leukemia expressed in the leukemic stem cell compartment

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1. Data processing and statistical assays

Flow cytometric (FCM) data were analyzed using Infinicyt software v.1.8 (Cytognos, Salamanca, Spain) or DIVA software (BD Biosciences, San Jose, CA, USA). Graphs were generated in GraphPad Prism version 5.04 for Windows (GraphPad Software, La Jolla California USA), Excel or PowerPoint (Windows). Images from gel electrophoresis, Western blotting and confocal microscopy were processed by ImageJ, Fiji and GIMP2 (free software packages available at Ghent University, Ghent, Belgium). Nucleotide sequence chromatograms were evaluated in BioEdit Sequence Alignment Editor for Windows (Ghent University). Reference mRNA sequences and annotations were derived from the University of California Santa Cruz (UCSC) Genome Browser Web-based tool using the GRCh38/hg38 Assembly. Post-sequencing alignment between samples and with UCSC reference sequences was performed in Vector NTI using the AlignX tool (Life Technologies).

Statistical calculations were performed in GraphPad Prism version 5.04 or MedCalc version 12.3.0.0 (Mariakerke, Belgium), with the exception of Chi Square test, for which MedCalc (version 18.11.3) was used. The Spearman's coefficient rank correlation coefficient was used to correlate cytotoxic killing rates with TARP transcript expression. Data were tested for normal distribution using the d'Agostino-Pearson test. One-way ANOVA with Tukey's Multiple Comparison post-test was performed to evaluate TARP transcript expressions between more than two groups. The Mann-Whitney U test was applied as a non-parametric test for independent samples from two groups. Paired sample T-test (Gaussian distribution) or Wilcoxon matched-pairs signed rank tests (non-Gaussian distribution) was used to compare expression levels before and after transduction, between different time points after transduction, and between LSCs and blasts sorted from the same patient. P-values calculated were two-tailed, and one, two, three or four asteriks are indicative the level of significance, set to 5% (0.05), 1% (0.01), 0.1% (0.001) and 0.01% (0.0001), respectively.

2. Material and methods

2.1. Culture, pulsing, human leukocyte antigen typing and fractionation of cell lines

Breast (BT-474, MCF-7) and prostate (PC3, LNCaP) adenocarcinoma cell lines were a gift from the Laboratory of Experimental Cancer Research (Ghent University Hospital, Ghent, Belgium). Four luciferase-expressing AML cell lines (HL60-luc, MOLM-13-luc, MV4;11-luc and OCI-AML-3-luc) were kindly provided by RWJ Groen and HJ Prins from the Cancer Center Amsterdam (CCA) (Vrije Universiteit Medical Center (VUmc), Amsterdam, the Netherlands). All remaining cell lines were purchased at ATCC or DMSZ. These included nine AML cell lines (HL-60, HNT-34, Kasumi-1, Kg-1a, MOLM-13, MONO-MAC6, MV4;11, OCI-AML3, THP-1), five B-ALL cell lines (E2A, REH, NALM-6, SEM, SUPB15), the CML cell line k562, the EBV-immortalized B-cell line JY and the T-ALL cell line HSB-2. Cell lines were grown in media according to supplier instructions at 37 °C in 5% or 7% CO_2 incubators. DMEM, IMDM and RPMI media (Invitrogen) were supplemented with 10% or 20% Fetal Calf Serum (FCS, Hyclone or ThermoFisher Scientific), 100 U/mL Penicillin/Streptomycin (10000 U/ml, Invitrogen) and 100 µg/mL L-Glutamine (200 mM, Invitrogen). For THP-1, medium was additionally supplied with 0.05 mM β-mercaptoethanol.

T2, a human leukocyte antigen (HLA)-A*0201-positive, TAP-deficient cell line, was used for *in vitro* pulsing with antigenic peptides (GenScript HK Limited (Hongkong), overview in Table S3). Per pulsing experiment, one million cells were incubated overnight (O/N) at 37 °C in IMDM supplemented with 1% human serum and 10 µg peptide solubilized in DMSO.

Human leukocyte antigen (HLA)-A sequencing was performed at the Red Cross (Mechelen, Belgium).

Subcellular compartmentalization of cell lines into nuclear and cytoplasmic fractions was performed according to the protocol of Gagnon et al.[1] Total nuclear and cytoplasmic RNA was resuspended in TRIzol and evaluated by qPCR, as described in 2.5.

2.2. Sorting strategy

All scatters were devoid of cell debris and doublets based on propidium iodide (PI) exclusion and FSC-H vs FSC-A, respectively. Sorting strategies were applied depending on the population of interest:

- Mononuclear cells (MNC) collected from AML patients and healthy controls were used to sort CD34+CD38+ and CD34+CD38- populations. CD34-postive AML scoring was done as previously defined[2, 3], identifying CD34-positive cases as those with > 1% of CD34+ blasts in the leukemic cells. If the number of CD34-positive cells concerned less than 50% of the total white blood cell (WBC) population, CD34-isolation was performed using the CD34 MicroBead Kit (Milteny). The immature myeloid compartment was defined by CD34, CD45 and scatter properties. CD34+CD38+ blasts and CD34+CD38- stem cells were gated as previously described[4]. Lymphocytes and fluorescence-minus-one (FMO) controls were used to determine CD38 expression cut-offs. Lymphocytes were sorted based on high CD45 expression and low SSC-A. Delineated cell populations were backgated on FSC-A/SSC-A and CD45/SSC-A scatter plots to exclude non-specific events, amongst other myeloid precursor populations. Sorted cells were collected in RPMI supplemented with 50% FCS and a post-sort purity of >90% was reached. Following, cells were spun down (10 min, 3000 rpm, 4° C) and resuspended in TRIzol for RNA and/or DNA extraction (see 2.5).
- Transgenic AML cell lines were sorted based on HLA-A*0201, eGFP or Zsgreen expression, depending on the transduction experiment. Sorted cells were collected in RPMI supplemented with 50% FCS, with post-sort purities described in 2.7.4. Sorted cells were further propagated in culture or resuspended in 700 µL TRIzol for RNA and/or DNA extraction (see 2.4).
- TCR-transgenic cytotoxic T-cells (CTLs) were sorted directly into 96 well-plates based on CD3/CD8 expression, in combination with being positive for mTCRab or eGFP for LV or RV transduced CTLs, respectively. Sorted cells were expanded on irradiated allogeneic feeder cell medium (see 2.8.4).

2.3. Micro-array profiling

CD34+CD38+ (n=4) and CD34+CD38- (n=3) cell fractions, and lymphocytes (n=4), were sorted from four *de novo* pedAML patients and used for profiling (two FLT3-ITD, two FLT3 WT, Table S1). As control, CD34+CD38+ (n=3) and CD34+CD38- (n=2) cells were sorted from cord blood (CB).

RNA was extracted using the miRNeasy Mini Kit (Qiagen) in combination with on-column DNase I digestion (RNase-Free DNase set, Qiagen) according to manufacturer's instructions. RNA quality and concentrations were measured by Agilent 2100 Bioanalyzer (Agilent) and Qubit (ThermoFisher Scientific), respectively. Mean RIN of all sorted fractions was 9.3 (95% CI 9.1-9.5). Cells were profiled on a custom 8x60K human Gene expression micro-array, containing probes for all human protein-coding genes with lncRNA content based on LNCipedia 2.1[5] (Biogazelle), as follows: 20 ng RNA was pre-amplified using the Complete Whole Transcriptome Amplification Kit (Sigma-Aldrich). Amplified RNA was subsequently labelled using the Genomic DNA ULS Labeling Kit (Agilent) and hybridized to

the array in combination with CGH blocking to reduce background signaling. Micro-arrays were analysed using an Agilent micro-array scanner and Feature Extraction software (v12.0). Probe intensities were background subtracted, quantile-normalised and log2-based probe intensities were calculated. A target was present if the log2 expression value exceeded the cut-off set at 6.75, based on the dark corner control probe value plus 1. Data processing was performed in R using packages EnhancedVolcano, ggplot2, plotMDS, Bioconductor and limma. Principle component analysis showed clustering of the sorted fractions according to their biological classes (Fig. S3 A, C) and according to their respective patient origin (Fig. S3 E).

2.4. RNA and DNA isolation, cDNA synthesis and (quantitative) PCR

Cell lines ($1x10^6$) and sorted cells (variable cell number) used for qPCR or FLT3-ITD mutational screening were resuspended in 700 µL TRIzol and frozen at -80 °C until further processing. DNA was extracted manually[6] or automatically using the QIAamp DNA Blood Mini Kit on a QIAcube platform (Qiagen) if cells were preserved in 75% ethanol. RNA was extracted using the miRNeasy Mini or Micro Kit (Qiagen) in combination with on-column DNase I digestion (RNase-Free DNase set, Qiagen) according to manufacturer's instructions. RNA concentrations were measured by Nanodrop (ThermoFisher Scientific) or Qubit RNA HS Assay (Invitrogen). cDNA synthesis was performed by Invitrogen SuperScript III or II Reverse Transcriptase (Invitrogen) according to the supplier`s recommendations.

All primers for TARP transcript evaluation were purchased at IDT Technologies (Table S3). The TARP transcript was previously described to initiate within the TRGJP intronic gene segment, followed by TRGC1 coding regions and an untranslated and poly(A) sequence at the end[7] (Fig. S2). Primers for TARP were chosen based on previous literature, different coding regions and the inclusion of the immunogenic $TARP_{4\text{-}13}$ coding region. For general TARP transcript evaluation, we used primers targeting the first exon of the TRGC gene segments, referred to as "TARP short". For sequencing, four different primer pairs were selected, targeting the entire (TARP long) or part of (TARP short) all TCRG exons, or exon 1 and exon 3 individually, as illustrated in Fig. S2. To elucidate the subcellular localization of TARP, we used primers directed against *MALAT1* (nuclear-retained non-coding RNA) and *TBP* (cytoplasmic TATA box binding protein). Reference genes for normalisation were *a priori* selected in a pilot study according to the state-of-the-art. The expression of eight housekeeping genes[8] (*GAPD, HMBS, HPRT1, RPL13A, SDHA, TBP, UBC, YWHAZ*) and Alu repeats[9] was investigated in 11 cell lines, chosen based on a broad genetic repertoire and pediatric origin. Adhering to a strict M-value ≤ 0.5 and V-value < 0.15[10], six out of eight housekeeping genes (ranged from highest to lowest stability: *TBP, GAPD, HPRT1, HMBS, SDHA* and *YWHAZ)* were advised for gene of interest normalisation. Due to practical considerations, the three most stable housekeeping genes *GAPD*, *HPRT1* and *TBP* (V-value 0.202, respective M-values 0.45, 0.45 and 0.57) were selected.

qPCR reactions were carried out in 96-well plates using 0.3 µM primers, Sso Fast Evagreen master mix (Bio-Rad), 2.38 ng cDNA and H_2O (Sigma-Aldrich) in a 10 µL reaction. Samples were run in duplicate by a 2-step real-time protocol (2 min 98 °C, followed by 45 cycles (98 °C 5 sec, 60 °C 20 sec)) on a Viia7 analyzer (ThermoFisher), combined with melting curve analysis (65 °C to 95 °C, gradually increasing with 0.5 °C/5 sec). Ct thresholds were automatically determined by the QuantStudio™ Real-Time PCR Software. Efficiency-corrected Ct values were calculated for each primer pair using the LinRegPCR software (AMC, University of Amsterdam, the Netherlands).

As TARP was previously defined as a truncated TCRG transcript[7], contaminating TCRγδ+ lymphocytes during sorting or the rare occurrence of cross-lineage TCRG locus recombinations in AML[11], could

impede interpretation. To exclude this, we performed DNA TCRG GeneScan analysis[12] and a qPCR with primers targeting VγI, VγII, VγIII and VγIV gene segments to exclude functional TCRG gene rearrangements (cycling protocol described above).

FLT3 mutational screening assay was developed by Molecular Diagnostics.be (Belgium). Briefly, a multiplex PCR was performed for fms-like tyrosine kinase receptor-3 (*FLT3*), nucleophosmin (*NPM1*) and CCAAT/enhancer binding protein alpha (*CEBPA*) mutations, followed by fragment analysis on an ABI 3130XL Genetic Analyzer using a ROX 500 internal standard (Applied BioSystems).

2.5. Post-qPCR analysis; gel electrophoresis, amplicon cloning, purification and sequencing

Amplicons generated by qPCR were run on 3% agarose gels for 2-3 h at 110 V, followed by staining of gels with 0.5 µg/mL ethidium bromide (EtBr) for 20 min. Images were captured using the bio-imager system Gel Doc XR+ (Bio-Rad). An overview of all agarose gel images is shown in Fig. S5. Following quick imaging, separate bands were isolated for gel extraction.

Sequencing of amplicons was performed at Eurofins GATC Biotech (Constance, Germany). Single bands visualized on gel were purified directly from 96-well plates using the Wizard SV PCR Clean-up system (Promega). If multiple bands were present, DNA was extracted from each band per amplicon (indicated as A, B or C; Fig. S5 A) using the MinElute kit (Qiagen) and measured by Nanodrop. If post-purification concentrations were insufficient to allow for accurate sequencing (< 20 ng/µL), the purified amplicons were ligated into a pCR®-Blunt vector according to manufactory instructions. Between 10-100 ng ligation product was transformed into One Shot® TOP10 *E. Coli* bacteria (for method, see 2.7.2). Following O/N incubation, 2-6 colony-forming units (CFU) were picked from each plate and incubated for 16 h at 37 °C in 5 mL Luria-Bertani (LB) broth whilst shaking for plasmid isolation. Day after, miniprep cultures were pelleted for 5 min at 2000 rpm and plasmids were isolated by QIAprep® miniprep kit (Qiagen), according to manufacturer's instructions. A restriction verification digest was done with *Eco*R1 (0.5 µL, 20000 U/mL, NEB) for each ligation product, followed by agarose gel electrophoresis, to select positive transformants.

2.6. Protein detection

2.6.1. Western blotting

Whole-cell protein extracts were prepared by washing 5 million cells three times with 10 mL ice-cold PBS. Cell pellets were resuspended in 1 mL RIPA buffer supplemented with Halt™ Protease and Phosphatase Inhibitor (1/100 diluted, both ThermoFisher Scientific), sheared fifteen times through a 27G needle, snap frozen and incubated on ice for 45 min with regular vortexing. Afterwards, protein lysates were spun down (13000 rpm, 10 min, 4 °C) and supernatant was frozen at -20 °C. Protein concentrations were measured at 560 nm using a GloMax Explorer (Promega) and PierceTM BCA Protein Assay Kit (ThermoFisher Scientific).

For immunoblotting, 40 µg protein extracts were denatured with 4x LDS and 10x DTT reducing agent and run (100 V, 70 min) on a 4-12% BIS-Tris Plus gels in 1x MES running buffer (Life Technologies). Proteins were transferred to a 0.2 µm pore PVDF (Invitrogen) membrane in 1x transfer buffer for 34 min at 20 V, and blocked with 5% milk TBS/0.05% Tween (TBS-T) for 2 h at room temperature (RT). Primary antibodies (anti-huTARP (TP1 a.k.a 1F8, abcam: 1/3000), anti-β-actin monoclonal (BA3R, Invitrogen: 1/2500) or anti-GFP (B-2, sc-9996, Santa Cruz Biotechnology: 1/500)) were diluted in 5%

milk TBS-T and incubated O/N at 4 °C whilst shaking. Blots were cut just above the 38 kDa ladder fragment to allow for simultaneous TARP staining and β-actin staining, avoiding reprobing. Following, blots were washed three times with TBS-T and incubated with HRP-linked sheep-anti-mouse secondary antibody (GE Healthcare Life Sciences: 1/5000) in TBS-T/5% milk for 1 h at RT. After washing, proteins were detected by SuperSignal West Femto Substrate (ThermoFisher Scientific) and an ImageQuant Las4000 with CCD camera. Percentage of TARP expression in knockdown cell lines was calculated relative to the respective mock and in respect to β-actin expression using ImageJ.

2.6.2. Confocal microscopy

Adherent cells were plated at $1x10^4$ cells/well in 96 optical glass plates (Perkin Elmer), followed by O/N incubation. Suspension cells were plated at $2x10^4$ cells/well on poly-L-Lysine hydrobromide (PLL, Sigma, 0.1 mg/mL)-coated wells at incubated for another 2 h at 37 °C. After incubation, cells were centrifuged (1200 rpm, 5 min), fixated and permeabilized with 4% paraformaldehyde for 15 min (RT) and 0.25% Triton X-100 for 10 min (RT), respectively. In between, wells were washed three times with PBS. Blocking was done at 4 °C O/N in PBS/1% BSA/5% goat serum (GS) whilst shaking. The day after, primary antibodies (anti-huTARP: 1/100, anti-HSP60 rabbit polyclonal (H-300, Santa Cruz Biotechnology): 1/50, anti-calnexin rabbit polyclonal (H-70, SC-1139, Santa Cruz): 1/75) were diluted in PBS/1% BSA/5% GS and incubated for 1 h at RT whilst shaking. After three washes with PBS/1%BSA, cells were incubated with secondary antibodies (goat anti-mouse IgG2a Alexa 647, chicken anti-rabbit IgG Alexa 488 or goat anti-mouse IgG1 Alexa488: 1/250) diluted in PBS/1% BSA for 1 h at RT in the dark whilst shaking. Cells were stained with TARP alone or combined with HSP-60 or calnexin. Cell nuclei were counterstained with DAPI and cells were visualized with a confocal fluorescence microscope combined with bright field imaging (Leica SPE).

2.7. Retroviral transduction of AML cell lines

2.7.1. Plasmids and glycerol stocks

The HLA-A*0201 plasmid was kindly provided by T. Mutis (CCA, VUmc, Amsterdam, the Netherlands) and used to generate HLA-A*0201-positive transgenic AML cell lines (see 2.7.4).

pDNR-Lib plasmids expressing end-sequenced TARP cDNA were used to isolate the TARP open-reading frame (ORF) cDNA, as previously described in prostate and breast adenocarcinoma[7] (TARP BC 105589, provided as glycerol stock, catalog# 6339-1, transOMIC technologies (Table S3)). The retroviral pMSCV-Puro IRES GFP (PIG) vector, a kind gift from Joshua Mendel lab, was used to express TARP under transcriptional control of the PGK promotor instead of puromycin, with GFP useful for isolation of positively transduced cells. To create pMSCV-TARP IRES GFP, the entire TARP ORF cDNA sequence was cloned into the pMSCV-PIG vector using *Eco*RI/*Nsi*I restriction enzyme sites. First, two PCR reactions were performed; one to create overlapping ends in the pMSCV-PIG vector with the TARP ORF cDNA (primers P1-P2, Table S3), and one to add *Eco*RI/*Nsi*I restriction enzyme sequences to each side of the TARP ORF cDNA (primers P3-P4, Table S3). PCR reactions were carried out using Phusion High-Fidelity PCR Master Mix (25 µL, New England Biolabs (NEB)), 0.2 µM primers (P1-P2 or P3-P4), 1.5 µL DMSO and 100 ng of pMSCV-PIG or pDNR-Lib plasmid DNA, with H_2O in a final 50 µL volume. The cycling protocol (98 °C 30 sec, followed by 35 cycles (98 °C 10 sec, 60 °C 30 sec, 72 °C 20 sec) and 10 min at 72 °C) was performed on a MasterCycler pro S (ManualShelf). Amplicon sizes were checked by agarose gel electrophoresis (0.9%, 40 min, 45 V) and purified from gel using the NucleoSpin® gel and PCR clean-up kit (Macherey-Nagel™). Second, an overlap PCR was performed to align P1-P2 and P3-P4 amplicons, and create a final 723 bp insert, in which the TARP ORF is under transcriptional control of the PGK promotor and flanked by *Eco*R1 and *Nsi*I restriction enzyme sites.

Fifty ng of P1-P2 and P3-P4 amplicons was added to a Phusion master mix (25 µL), containing DMSO (1.5 µL) and H_2O (final volume 50 µL) and amplified without primers (98 °C 30 sec, 10x (98 °C 10 sec, 60 °C 30 sec, 72 °C 20 sec). Then, P1-P4 primers (0.2 µM) were added and amplification continued for another 25 cycles. Amplicon size of the final insert cDNA was checked by gel electrophoresis, followed by excision and purification from gel. Third, 700 ng insert cDNA and pMSCV-PIG vector were double digested using restriction enzymes *Eco*R1 (0.5 µL, 20 000 U/mL, NEB #R3101) and *Nsi*I (1 µL, 10 000 U/mL, NEB #R0127S), 2 µL CutSmart® buffer (NEB) and H_2O in a 20 µL reaction volume. Digest mixtures were incubated for 2 h at 37 °C, followed by 20 min at 80 °C. Digested products were run by agarose gel electrophoresis, and after purification, the cutted insert cDNA and backbone vector were ligated O/N at 20 °C (3:1 molar ratio) using T4 DNA ligase, 10x buffer (NEB) and H_2O (final volume 30 µL). The final ligated vector, containing the TARP ORF cDNA, is referred to as pMSCV-TARP-IRES-GFP, and was subsequently used for transformation (see 2.9.2.).

To generate TARP knockdown (KD) AML cell lines, three shERWOOD UltramiR short hairpin (shRNA) targeting TARP, and one non-targeting shRNA, were purchased as glycerol stocks at transOMIC technologies (TRHSU2000-445347, Table S3). The pLMN backbone allowed for selection of retroviral integration based on Zsgreen fluorescent expression and Geneticin-supplemented medium (G418 Sulfate, ThermoFisher Scientific).

2.7.2. Plasmid transformation and isolation

pDNR-Lib and pLMN-shRNA 1, 2, 3 and NT plasmids were provided as glycerol stocks. Bacteria were gently scratched from the glycerol stock into LB broth supplemented with chloramphenicol (50 µg/mL, Sigma-Aldrich) and ampicillin (100 µg/mL, Sigma-Aldrich) for pDNR-Lib and pLMN plasmids, respectively. Bacteria were cultured at 33 °C whilst shaking, and after 16 h, bacterial cultures were pelleted (5 min, 2000 rpm) and plasmids were isolated by maxiprep (ZymoPURE™ Plasmid Maxiprep Kit, ZymoResearch) or midiprep (NucleoBond® Xtra Midi, Machery-Nagel), according to manufacturer's instructions.

For HLA-A*0201, pMSCV-TARP-IRES-GFP and pMSCV-PIG vectors were transformed into Stbl3 bacteria (ThermoFisher Scientific) according to manufacture instructions. Briefly, 10-100 ng plasmid DNA, or 1-5 µL H_2O (negative control), were added to 25 µL bacteria. After gentle mixing, tubes were incubated on ice for 30 min. Bacteria were heat-shocked in a 42 °C water bath for 30 sec, and placed on ice. SOC outgrowth medium (975 µL heated at 37 °C, NEB) was added to each tube and vigorously shaken at 37 °C for 1 h at 225 rpm. Afterwards, 100 µL of each bacterial suspension was plated on ampicilline LB agar plates (100 µg/mL) and incubated O/N at 37 °C. Day after, CFU were counted in respect to the control plate, and between 1-6 CFU were further grown at 33 °C in ampicilline-supplemented LB for 16 h whilst vigorous shaking. Day after, bacterial suspensions were centrifuged (5 min, 2000 rpm) and plasmids were isolated by midiprep (NucleoBond® Xtra Midi, Machery-Nagel) or QIAprep® miniprep (Qiagen).

2.7.3. Virus production

Retrovirus encoding MHC-I HLA-A*0201, transgenic TARP for overexpression (pMSCV-TARP-IRES-GFP) and TARP-targeting shRNA for knockdown (pLMN-shRNA1, 2 and 3), together with non-targeting mock controls (pMSCV-PIG and pLMN-NT shRNA, respectively), were generated as follows: HEK 293T packaging cells (Clontech) were seeded at 70% density in 10 cm dishes pre-coated with 0.1% gelatin, and incubated O/N in DMEM supplemented with 10% FCS at 37 °C (5% CO_2). The day after, medium was refreshed 1 h before transfection, and a 25 µg DNA mix was prepared in 450 µL H_2O (3:1:1 ratio; transfer plasmid:pAmpho (Clontech):pHit60 (Roche)). Cells were calcium phosphate

transfected (50 µL $CaCl_2$, 500 µL 2XHBS buffer, both by InVitrogen) and incubated O/N at 37 °C (5% CO_2). Medium was refreshed after 16 h, and viral supernatant was collected 48 h and 72 h post-transfection. Viral supernatant was centrifuged to remove cell debris (1800 rpm, 5 min) and immediately used for transduction or frozen in 500 µL aliquots at -80 °C.

2.7.4. Viral transduction

Retroviral transduction of AML cell lines in order was performed as follows: 24-well plates were coated with retronectin (6 µg/well, TaKaRa) O/N at 4 °C. Coated wells were blocked with PBS/2% BSA (30 min, RT) and washed with PBS. Target cells were plated in medium at a target-dependent multiplicity of infection ratio, ranging between 0.5-0.75 $x10^6$ cells/well, and virus supernatant was added in a 2:1 virus:target ratio in the presence of polybrene (6 µg/well, Sigma-Aldrich). Plates were spinoculated for 90 min at 2300 rpm (32 °C) and incubated at 37 °C (5% CO_2). The next day, transduction was repeated with the exception that after eight hours half the medium was refreshed after which the plates were incubated for 72 h at 37 °C (5% CO_2). The number of biological replicates, transduction efficiency and post-transduction selection method differed between transduction experiments, and are discussed below.

Six AML cell lines (HL-60, Kg-1a, MOLM-13, HL-60-Luc, MOLM-13-Luc and MV4;11-Luc,) were transduced to express HLA-A*0201 MHC-I molecules. Transduction was performed in triplicate per cell line and transduction efficiency ranged between 6.2-89.2 %. Positively transduced cells were selected via HLA-A*0201 APC antibody staining and EasySep™ APC Positive Selection Kit (STEMCELL Technologies) according to manufacturer's instructions. After EasySep selection, HLA-A*0201 transduced cells were on average 96% of the total viable cell population. TARP transcript levels were not influenced by HLA-A*0201 transduction (not shown). The number of HLA-A*0201 expressing cells remained >96% during short-term culture (1 month) but diminished over a period of six months for HL-60 and Kg-1a (60.0% and 55.7%, respectively).

Two HLA-A*0201-positive AML cell lines, OCI-AML3 and THP-1 with low and high endogenous TARP expression respectively, were transduced to overexpress TARP (OE), alongside a mock. Transduction was performed in triplicate and transduction efficiency was evaluated based on eGFP expression after 72 h and 144 h using FCM (range 16.0-57.9%). Efficiencies were shown to be highly comparable between replicates, without significantly difference between both time points ($P>0.05$, Wilcoxon signed-rank test). Transgenic TARP OE and mock OCI-AML3 and THP-1 replicates were pooled, sorted at >90% purity, and eGFP expression sustained >92% during culture afterwards. Post-transduction TARP transcript and protein expression levels were measured after sorting (Fig. S6).

Four TARP-high AML cell lines (HL-60, Kg-1a, MV4;11 and THP-1) were transduced with three different TARP-targeting shRNA, alongside a non-targeting shRNA as mock (single replicate). Seventy-two hours post-transduction, cells were collected and cultured in Geneticin-supplemented medium to increase the percentage of positive retroviral integrants. Geneticin sensitivity of each cell line was determined before transduction, with optimal concentrations of 400 µg/mL, 500 µg/mL, and 300 µg/mL for HL-60, MV4;11 and THP-1, respectively (Kg-1a was unsuccessful). After two weeks on selection, positively transduced cells showed an average Zsgreen expression of 39% (95% CI 27.0-49.6%), 53% (95% CI 49.6-56.4%) and 84% (95% CI 81.5-86.5%) for HL-60, MV4;11 and THP-1, respectively. As selection was not possible for Kg-1a, a much lower percentage of positively transduced cells (mean 6%, 95% CI 2.2-9.3%) was obtained. KD and mock cell lines were subsequently sorted based on Zsgreen expression and further cultured in Geneticin-supplemented medium. Zsgreen expression post-sort was >91 % (95% CI 88.8 - 93.8), except for Kg-1a (mean 24%, 95% CI 15.8-31.5%), and remained stable during culture. Post-transduction TARP transcript (Fig. S9) and protein (Fig. 3C) expression values were measured after sorting.

2.8. Retro- and lentiviral transduction of cytotoxic T-cells (CTLs)

2.8.1. Plasmids

The pBMN(TARP$_{4\text{-}13}$-TCR) target plasmid and pLP1, pLP2 and pLP/VSVG helper plasmids for LV transduction were provided by M. Essand.

The pBMN(TARP$_{4\text{-}13}$-TCR) plasmid encodes a TCRA8-T2A-TCRB12 sequence directed against the HLA-A*0201-restricted synthetic TARP peptide TARP(P5L)$_{4–13}$[13, 14]. However, in our setting, LV transduction appeared to be inconvenient. To start, selection of positively transfected packaging cells was hampered by the lack of an antibiotic resistance or a fluorescent marker in the pBMN backbone. Consequently, transduction efficiencies topped at 10 %, and mouse TCR constant domain (mTCRab) antibody staining was required for the evaluation of transduction efficiencies and sorting out positively transduced CTLs. In addition, the backbone plasmid was not suitable for the generation of non-targeting viral particles and cytotoxic killing experiments consequently lacked mock CTLs to correct for non-TARP mediated lysis. Therefore, the TCR coding region was amplified from the pBMN vector and cloned into the retroviral LZRS-IRES-GFP (LIE) vector.

To this end, primers were designed to amplify the TARP$_{4\text{-}13}$-TCR sequence from the pBMN(TARP$_{4\text{-}13}$-TCR) vector, which incorporate the restriction enzyme sites *Bam*HI and *Xho*I present in the LIE plasmid (acceptor), including a Kozak sequence. These primers (P5 and P6 (0.5 µM), Table S3) were added to 20 ng pBMN plasmid DNA in a 20 µL Phusion High-Fidelity reaction mixture (5x Phusion Green buffer, Phusion hot start II Polymerase (2 U/L, 0.2 µL), dNTPS (0.4 µL, 10 mM) (all by ThermoFisher Scientific), and H_2O). PCR was run in eightfold on a Veriti 96-Well Thermal Cycler (ThermoFisher Scientific) (cycling protocol 98 °C 1 min, followed by 40 cycles (98 °C 7 sec, 69 °C 15 sec, 72 °C 10 sec) and 7 min at 72 °C). Amplicons were pooled, checked by gel electrophoresis, and the TARP$_{4\text{-}13}$-TCR sequence with LIE-overlapping ends, hereafter referred to as insert, was purified using the QIAquick Gel Extraction Kit (Qiagen). Subsequently, both insert (150 ng) and LIE backbone vector (75 ng) were double digested with *Bam*HI (0.5 µL, 20 000 U/mL, NEB #R0136S) and *Xho*I (0.5 µL, 20 000 U/mL, NEB #R0146S) restriction enzymes and ligated at 50 °C for 1 h in 10 µL Gibson Assembly® master mix (NEB). The resulting ligated vector, referred to as TARP-TCR LIE plasmid, was used for transformation and plasmid isolation (see 2.10.2).

In addition, we used the empty LIE vector as control, hereafter defined as mock. As positive control, we used an in-house created CMV-TCR encoding LIE vector, using a TCR-sequence provided by Leiden University Medical Center (Leiden, Netherlands) as previously described[15], defined as CMV-TCR LIE plasmid.

2.8.2. Plasmid transformation and isolation

Twenty-five µL DH10B bacteria (NEB) were thawed on ice, and per reaction, 10-100 ng plasmid DNA (pBMN(TARP$_{4\text{-}13}$-TCR), pLP1, pLP2, pLP/VSVG and LIE plasmids encoding TARP-TCR, CMV-TCR or empty) was added, next to H_2O (15 µL) as control. Transformation was performed as described in 2.7.2. Afterwards, 100 µL of each bacterial suspension was plated on ampicilline LB agar plates (100 µg/mL) and incubated O/N at 37 °C. Day after, CFU were counted in respect to the control plate, and further grown for 16 h at 33 °C in ampicilline-supplemented LB (100 µg/mL) whilst shaking. Day after, bacterial suspensions were spinoculated (5 min, 2000 rpm) and plasmids were isolated by midiprep (NucleoBond® Xtra Midi, Machery-Nagel) or miniprep (QIAprep® Miniprep), according to manufacturer's instructions.

2.8.3. Virus production

TARP-TCR encoding lentivirus was produced by seeding HEK293FT packaging cells ($6.6x10^6$) in T175 flasks containing DMEM with 10% FCS, followed by O/N incubation at 37 °C (5% CO_2). Day after, medium was refreshed 3 h before transfection and a 20 µg DNA mix (2:1:1:1 ratio; pBMN:pLP1:pLP2:pLPVSVG) was prepared. Cells were transfected using jetPEI/NaCl (PolyPlus transfection) and 16 h later, medium was refreshed with reduced serum (1% FCS) DMEM. Viral supernatant was collected 48 h and 72 h post-transfection, immediately placed on ice, spun down (1500 rpm, 7 min, 4 °C) and filtered through a 45 µM low-binding PVDF filter. Subsequently, the filtered supernatant was 10X concentrated using Amicon® Ultra-15 Centrifugal Filters (EMD Millipore™, ThermoFisher Scientific) and aliquoted at -80 °C until use.

TARP-TCR LIE, CMV-TCR LIE or mock encoding retrovirus were produced by seeding phoenix-A packaging cells ($1x10^6$) in 6 cm dishes containing IMDM with 10% FCS, followed by O/N incubation at 37 °C (7% CO_2). Medium was refreshed day after and cells were calcium-phosphate transfected (36 µL $CaCl_2$ (2M) and 300 µL 2XHBS buffer, both by InVitrogen) with 10 µg plasmid DNA after the addition of 1 µL chloroquine (200 mM, Sigma). Medium was refreshed 16 h post-transfection, and after 72 h, transfected packaging cells were transferred to medium supplemented with puromycin (2 µg/ml). Puromycin-supplemented medium was exchanged every three days, and after two weeks, viral supernatant (eGFP expression >99%) was collected on ice, followed by double spinoculation (1500 rpm, 7 min, 4 °C) and storage at -80 °C until further use.

2.8.4. Viral transduction

Buffy coats for CTL isolation were obtained from the Red Cross (Mechelen, Belgium) and derived from HLA-A*0201-negative donors (n=4, used for LV transduction) or HLA-A*0201 positive donors (n=2, used for RV transduction). MNC were purified from buffy coats using standard Ficoll Density gradient (Axis-shield). CD8-positive CTLs were isolated using CD8-biotine (OKT8) and streptavidin MicroBeads (Milteny Biotech) in PBS supplemented with 2 mM EDTA and 2% FCS. Before transduction, CD8+ CTLs were stimulated with CD3/CD28 T-cell activation Dynabeads (Life Technologies) in the presence of IL-12 (10 ng/mL) and IL-2 (30 IU/mL), or ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator (Stem Cell Technologies) supplemented IL-2 (20 IU/mL), respectively, following manufacturer's guidelines. Seventy-two hours post-stimulation, CD8+ CTLs were collected and plated in retronectin-coated, 2% BSA-PBS blocked 24-well plates. For LV TARP-TCR transduction, $2x10^5$ stimulated CD8+ CTLs were incubated with 10X concentrated TARP-TCR encoding lentivirus per well (1:1 ratio) in the presence of polybrene (4 µg/well) and IL-2 (40 IU/mL). For RV transduction, $2.5x10^5$ stimulated CD8+ CTLs were incubated with TARP-TCR LIE, CMV-TCR LIE or LIE empty retrovirus supplemented with IL-2 (10 IU/mL). Plates were centrifuged for 90 min (2300 rpm, 32 °C) and afterwards incubated at 37 °C (5% CO2). Two rounds of transduction were performed and cells were collected 48 h after the second transduction hit. Transduction efficiencies were by average 5.0% (95% CI 1.88-11.9%) for LV transduction and 58.9% (95% CI 38.3-79.5%) for RV transduction.

Positively transduced CTLs were cell-sorted, as described in 2.3, and expanded on irradiated feeders. Feeders consisted out of 40-Gy irradiated MNC isolated from buffy coats from healthy donors and 50-Gy irradiated JY cells (ratio 10:1) in the presence of 2 µg/mL PHA and 40 IU/mL IL-2. RV transduced CTLs were expanded once whereas LV transduced CTLs required two rounds. Sort purities ranged below 90% for LV transduced CTLs, but mTCRab expression remained stable during feeder expansion (median 50.8%, 95% CI 39.1-60.1%). RV transduced CTLs showed post-sort purities >95%

with stable eGFP expression during feeder expansion (median 76.2%, 95% CI 62.3-89.0%). Reactivity was assessed 10 days post-expansion at earliest using freshly collected CTLs or thawed CTLs (O/N incubation at 37 °C in IMDM with 10% FCS and 50 IU/mL IL-2).

2.9. Cytotoxicity assays

2.9.1. Flow cytometry-based assays

2.9.1.1. Cytokine release assay

Feeder-expanded CTLs were incubated with violet tracer (VT)-labelled targets in 96-well U-bottom plates for 1 h (E/T ratio 2/1), followed by the addition of GolgiStop™ (1/750, BD Biosciences)[16]. Effector CTLs incubated in medium or with JY cells (untreated wells) or aCD3(home-made)/aCD28 (BD) coated wells were used as negative (NC) and positive control (PC), respectively. Fifty thousand target cells were added to each well, and conditions were performed in duplicate (quadruplicate for NC and PC). After an additional 16 h of stimulation, cells were harvested into 96-well V-bottom plates and Flow-Count™ Fluorospheres (1:20 diluted, Beckman Coulter) were added to each well. Bead/target/effector cell mixtures were surface stained for 30 min (CD13 or CD33/CD34/CD45 for target cells and CD3/CD8/CD45 for CTLs), washed with PBS, and subsequently fixated and permeabilized (Fixation and Cell Permeabilization Kit, ThermoFisher) followed by intracellular IFN-γ and IL-2 staining. Cytokine release was calculated based on IFN-γ/IL-2 double positive cell populations within the CD3+/CD8+ compartment, evaluated compared to FMO controls, and expressed relatively to the positive control (PC).

2.9.1.2. Cytotoxicity lysis assay

VT-labelled targets were incubated in medium (untreated) or with CTLs (killing) in 96-well U-bottom plates for 48 h (LV, E/T ratio 10/1) or 24 h (RV, E/T ratio 1.25/1, 2.5/1 or 5/1, depending on the experiment). Ten thousand target cells were added to each well, and conditions were performed at least in duplicate (quadruplicate for untreated controls). After incubation, cells were harvested into 96-well V-bottom plates and Flow-Count™ Fluorospheres (1:20 diluted, Beckman Coulter) were added to each well. Bead/target/effector cell mixtures were cell-surface stained as described in 2.11.1.1. In case of HLA-A*0201-positive targets and LV TARP-TCR transduced CTLs, cell mixtures were additionally surface stained with HLA-A2 and mTCRab antibodies, respectively. To evaluate killing, Flow-Count-equalized absolute target cell numbers were calculated and lysis was determined as [1 − (viable target cells (killing)/viable target cells (untreated)] × 100%. For RV transduced CTLs, killing percentages were corrected versus the killing observed by mock CTLs.

2.9.2. ^{51}Chromium release assay

Target cells ($0.5x10^6$) were labelled with 50 µCi ^{51}Chromium (Perkin Elmer, NEZ030005MC) during 90 min at 37 °C (5 % CO_2), and washed twice with 7.5 mL IMDM with 10% FCS before incubation with CTLs. Different E/T ratios were used for LV (5/1, 10/1, 20/1 and 50/1) and RV (1/1, 5/1, 10/1 and 20/1) transduced CTLs and all killing conditions were performed in duplicate. Each target was incubated in medium with or without 2% Triton (Sigma-Aldrich) as PC or NC, respectively (quadruplicate analysis). Following 4 h incubation (37 °C, 5% CO_2), supernatant was harvested and measured in optiphase HISAFE 3 (1:3 ratio) by a 1450 LSC&Luminescence Counter (both by Perkin Elmer). Specific lysis was calculated as [(release (killing) – release (NC))/(release (PC) – release (medium))] x100%.

2.9.3. Bioluminescence imaging-based cell lysis assays

Luciferase (Luc)-positive cell lines were incubated with medium (untreated) or with LV TARP-TCR engineered CTLs (killing) in 96-well U-bottom plates for 8 h, 24 h, 48 h and 56 h (E/T ratio 10/1). Ten thousand target cells were added to each well, and conditions were performed at least in duplicate (quadruplicate for untreated controls). After incubation and adding beetle luciferin (125 µg/mL), luciferase emission (relative light units, RLU) was determined as a measure of target cell viability within 10 min on a GloMax196 Microplate Luminometer (both by Promega) at 420 nm. Baseline luminescence, measured at time point zero, was set at 100% viability for each target. Lysis was calculated as [(RLU (untreated) – RLU (killing)/RLU (untreated)] and specific lysis was calculated for TARP-high cell lines based on the difference between killing of HLA-A*201-positive versus WT targets.

3. Supplemental Tables

		Median (Range)
Age, years		14 (10-15)
WBC count, x 10^9/L		79 (58.1-118)
Morphological blast count		
BM, %		88 (34-96)
PB, %		74 (38-78)
		N (%)
Gender		
F		3 (75%)
M		1 (25%)
CBF leukemia		2 (50%)
WT1 overexpression		2 (50%)
Mutation status	NPM1	0
	FLT3-ITD	2 (50%)

Table S1. Characteristics of *de novo* pedAML patients used for sorting CD34+CD38+ and CD34+CD38- cell fractions and micro-array profiling.

All four pedAML patients were diagnosed in Belgium, classified as standard risk and included for treatment in the Dutch-Belgian (DB AML-01) protocol. Morphological evaluation according to the FAB classification categorized patients as M0 (n=1), M2 (n=1) or M4 (n=2). Bone marrow with CD34+ leukemic cells was used for sorting. *WT1* overexpression was interpreted in regard to in-house or published (Cilloni et al. 2009) cut-offs. CBF-positive leukemias comprised AML with t(8;21)(q22;q22) (n=1) and inv(16)(p13q22) (n=1). Abbreviations are explained in the legend of Table 1.

Antibody	**Clone**	**Supplier**	**Category no.**
CD3 BV421	SK7	BD Biosciences	563798
CD3 PE-Cy7	UCHT1	eBioscience	25-0038-42
CD8 APC	SK1	Biolegend	344722
CD8a APC FIRE750	SK1	Biolegend	344746
HLA-A2 FITC	BB7.2	BD Biosciences	343304
HLA-A2 PE	BB7.2	BD Biosciences	558570
HLA-A2 APC	BB7.2	BD Biosciences	561341
IFNγ PE	25723.11	BD Biosciences	340452
IL-2 APC	MQ1-17H12	Biolegend	500310
TCR α/β PE	BW242/412	Miltenyi	130-091-236
CD13 PE	L138	BD Biosciences	347406
CD33 PE	P67.6	BD Biosciences	345799
CD34 PerCP-Cy5.5	8G12	BD Biosciences	333146
CD38 APC-H7	HB7	BD Biosciences	656646
CD45 PacO	HI30	Invitrogen	MHCD4530
LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit	/	ThermoFisher Scientific	L10119
Anti-Mouse TCR β Chain (mTCRαβ)	H57-597	BD Biosciences	561081

Table S2. Overview of antibodies.

Antibody concentrations were applied as recommended by the supplier. No. indicates number.

Name	Type	Nucleic acid (primers/shRNA) or amino acid (peptides) sequence s	
TARP long	primers TARP	GATAAACAACTTGATGCAGATGTTTCC	TTATGATTTCTCTCCATTGCAGCAG
TARP short	primers TARP	ACGGTGCCAGAAAAGTCACTGG	GGGAAACATCTGCATCAAGTTGTTTAT
TARP exon 1	primers TARP	GATAAACAACTTGATGCAGATGTTTCC	CTCAAGAAGACAAAGGTATGTTCCAGC
TARP exon 3	primers TARP	ATACACTACTGCTGCAGCTCACAAACA	TTATGATTTCTCTCCATTGCAGCAG
TCR V_γI-C_γ	primers TRCV(J)C	AACTTGGAAGGGRGAACRAAGTCAGTC	GGGAAACATCTGCATCAAGTTGTTTAT
TCR V_γII-C_γ	primers TRCV(J)C	CGGCACTGTCAGAAAGGAATC	GGGAAACATCTGCATCAAGTTGTTTAT
TCR V_γIII-C_γ	primers TRCV(J)C	TTGGACTTGGATTATCAAAAGTGG	GGGAAACATCTGCATCAAGTTGTTTAT
TCR V_γIV-C_γ	primers TRCV(J)C	TTGGGCAGTTGGAACAACCTGAAA	GGGAAACATCTGCATCAAGTTGTTTAT
MALAT1	primers localisation	GGATTCCAGGAAGGAGCGAG	ATTGCCGACCTCACGGATTT
GAPD	primers housekeeping gene	GGCATGGACTGTGGTCATGAG	GGCATGGACTGTGGTCATGAG
HPRT1	primers housekeeping gene	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
TBP	primers housekeeping gene/localisation	CGGCTGTTTAACTTCGCTTC	CACACGCCAAGAAACAGTGA
P1	primers for pMSCV-TARP-IRES-GFP	ATCTCTCGAGGTTAACGAAT	/
P2	primers for pMSCV-TARP-IRES-GFP	/	GAAGAAAAATAGTGGGCTTGGGGGAAACATCTG CATCGAAAGGCCCGGA
P3	primers for pMSCV-TARP-IRES-GFP	ATGCAGATGTTTCCCCCAAG	/
P4	primers for pMSCV-TARP-IRES-GFP	/	AATACGTAATGCATATGCATTCATGGTGTTCCCCT
P5	primers for TARP4-13-TCR LIE	GGGTGGACCATCCTCTAGACTGCCGGATCCGCCACCA TGCTGCTGCTG	/
P6	primers for TARP4-13-TCR LIE	/	CGTAGCGGCCGCGGCGCGCCGGCCCTCGAGTTAG CTGTTCTTCTTCTTCACCATGG
FLT3-ITD	primers FLT3	AGCAATTTAGGTATGAAAGCCAGC (FAM)	gtttcttCATCTTTGTTGCTGTCCTTCCAC
pLMN shRNA 1 (ULTRA-3326563)	Knockdown shRNA 1	TGCTGTTGACAGTGAGCGCAAAGATGCAAATGATACACTATAGTGAAGCCACAGATGTATAGTGTATCATTT GCATCTTTTTGCCTACTGCCTCGGA	
pLMN shRNA 2 (ULTRA-3326566)	Knockdown shRNA 2	TGCTGTTGACAGTGAGCGCCACAAACACCTCTGCATATTATAGTGAAGCCACAGATGTATAATATGCAGAGG TGTTTGTGAT GCCTACTGCCTCGGA	
pLMN shRNA 3 (ULTRA-3326567)	Knockdown shRNA 3	TGCTGTTGACAGTGAGCGCCACAATGGATCCCAAAGACAATAGTGAAGCCACAGATGTATTGTCTTTGGGAT CCATTGTGAT GCCTACTGCCTCGGA	
pLMN NT shRNA	Knockdown shRNA mock	TGCTGTTGACAGTGAGCGaaggcagaagtatgcaaagcatTAGTGAAGCCACAGATGTAatgctttgcatacttctgcctgT GCCTACTGCCTCGGA	
pDNR-Lib TARP	plasmid cDNA for transgenic TARP expressing cell lines (TARP ORF cDNA indicated in bold: 176 nucleotides)	GGGGGTTGGGCAAAAAAATCAAGGTATTTGGTCCCGGAACAAAGCTTATCATTACAGACAAATAAAACACC AAAAGCTTTAAGTTATTTGATTTGTGGAGCAACAGAACTTGTTATGAGCAAAATGAACCAGGACTGGAACCC TGGTCTTTTGAGAATCCCAGACCACCAGAATTTGAAGAACTCAGGGAAACTGAATTAGAGTTTTTGATATGG ACTGAATCACTGTGGAATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTTGTTGTCACAGATAAACAA CTTG**ATGCAGATGTTTCCCCCAAGCCCACTATTTTTCTTCCTTCAATTGCTGAAACAAAGCTCCAGAAGGCTG GAACATACCTTTGTCTTCTTGAGAAATTTTTCCCTGATGTTATTAAGATACATTGGCAAGAAAAGAAGAGC AACACGATTCTGGGATCCCAGGAGGGGAACACCATG**AAGACTAACGACACATACATGAAATTTAGCTGGT TAACGGTGCCAGAAAAGTCACTGGACAAAGAACACAGATGTATCGTCAGACATGAGAATAATAAAAACGG AGTTGATCAAGAAATTATCTTTCCTCCAATAAAGACAGATGTCATCACAATGGATCCCAAAGACAATTGTTCA AAAGATGCAAATGATACACTACTGCTGCAGCTCACAAACACCTCTGCATATTACATGTACCTCCTCCTGCTCC TCAAGAGTGTGGTCTATTTTGCCATCATCACCTGCTGTCTGCTTAGAAGAACGGCTTTCTGCTGCAATGGAGA GAAATCATAACAGACGGTGGCACAAGGAGGCCATCTTTTCCTCATCGGTTATTGTCCCTAGAAGCGTCTTCTG AGGATCTAGTTGGGCTTTCTTTCTGGGTTTGGGCCATTTCAGTTCTCATGTGTGTACTATTCTATCATTATTGTAT AACGGTTTTCAAACCAGTGGGCACACAGAGAACCTCACTCTGTAATAACAATGAGGAATAG	
$TARP_{4-13}$	peptide for pulsing	FPPSPLFFFL	
$TARP(P5L)_{4-13}$	peptide for pulsing	FLPSPLFFFL	
$TARP(V28L)_{27-35}$	peptide for pulsing	FLFLRNFSL	
INF_{58-66}	peptide for pulsing	GILGFVFTL	
CMV pp65	peptide for pulsing	NLVPMVATV	

Table S3. Overview of the nucleic acid sequences of primers and plasmids, and amino acid sequences of peptides.

Nucleotide sequences are shown from 5' to 3' direction and the genomic location of the first eight primer pairs is illustrated in Fig. S2. Primers were developed in-house or adapted from the MolecularDiagnostics.be assay (unpublished) or previous reports. Forward (left column) and reverse (right column) sequences from one primer pair are shown in the same row. For peptides, N-terminal anchor residues were modified into leucine (L) for TARP4-13 (proline (P) at position 5) and $TARP_{27-35}$ (valine (V) at position 28) peptides, to increase the ability of the peptide-MHC complex to activate cytotoxic T-cells.

TARP indicates T-cell receptor γ chain alternate reading frame protein; INF, influenza A; CMV, cytomegalovirus. Remaining abbreviations are explained in the main text or previous legends.

Type of sample	Sample code / cell line name	Method	Result
adult AML blast	adult AML 2	DNA TCRG GeneScan analysis / qPCR	NP/neg
adult AML blast	adult AML 6	DNA TCRG GeneScan analysis / qPCR	NP/neg
adult AML blast	adult AML 8	DNA TCRG GeneScan analysis / qPCR	NP/neg
adult AML blast	adult AML 12	DNA TCRG GeneScan analysis / qPCR	NP/neg
adult AML blast	adult AML 13	qPCR	neg
adult AML blast	adult AML 14	qPCR	neg
adult AML blast	adult AML 15	qPCR	neg
adult AML blast	adult AML 16	qPCR	neg
adult AML LSC	adult AML 12	qPCR	neg
AML cell line	HL-60	DNA TCRG GeneScan analysis / qPCR	NP/neg
AML cell line	HNT-34	DNA TCRG GeneScan analysis / qPCR	NP/neg
AML cell line	Kasumi-1	qPCR	neg
AML cell line	Kg-1a	DNA TCRG GeneScan analysis / qPCR	NP/neg
AML cell line	MOLM-13	DNA TCRG GeneScan analysis / qPCR	NP/neg
AML cell line	MONO-MAC6	DNA TCRG GeneScan analysis / qPCR	NP/neg
AML cell line	MV4;11	DNA TCRG GeneScan analysis / qPCR	NP/neg
AML cell line	OCI-AML3	qPCR	neg
AML cell line	THP-1	qPCR	neg
B-ALL cell line	E2A	qPCR	neg
B-ALL cell line	NALM-6	qPCR	neg
B-ALL cell line	REH	qPCR	neg
B-ALL cell line	SEM	qPCR	neg
B-ALL cell line	SUP-B15	qPCR	neg
breast adenocarcinoma cell line	BT-474	DNA TCRG GeneScan analysis / qPCR	NP/neg
breast adenocarcinoma cell line	MCF7	qPCR	neg
CB blast	Pool CB	qPCR	neg
CB blast	pooled CB 1	qPCR	neg
CB blast	pooled CB 2	qPCR	neg
CB HSC	Pool CB	qPCR	neg
CB HSC	pooled CB 1	qPCR	neg
CB HSC	pooled CB 2	qPCR	neg
NBM blast	NBM 3	DNA TCRG GeneScan analysis / qPCR	NP/neg
NBM blast	NBM 5	qPCR	neg
NBM HSC	NBM 3	qPCR	neg
NBM HSC	NBM 5	qPCR	neg
pedAML blast	pedAML 1	qPCR	neg
pedAML blast	pedAML 11	DNA TCRG GeneScan analysis / qPCR	NP/neg
pedAML blast	pedAML 2	DNA TCRG GeneScan analysis / qPCR	NP/neg
pedAML blast	pedAML 3	qPCR	neg
pedAML blast	pedAML 4	qPCR	neg
pedAML blast	pedAML 6	DNA TCRG GeneScan analysis / qPCR	NP/neg
pedAML LSC	pedAML 11	DNA TCRG GeneScan analysis / qPCR	NP/neg
pedAML LSC	pedAML 2	DNA TCRG GeneScan analysis / qPCR	NP/neg
pedAML LSC	pedAML 4	qPCR	neg
pedAML lymfo	pedAML 4	Heteroduplex	polyclonal
pedAML lymphocytes	pedAML 2	DNA TCRG GeneScan analysis / qPCR	polyclonal/pos
pedAML lymphocytes	pedAML 4	qPCR	pos
prostate adenocarcinoma cell line	LNCaP	DNA TCRG GeneScan analysis / qPCR	NP/neg
prostate adenocarcinoma cell line	PC3	qPCR	neg

Table S4. Exclusion of functional TCRG recombinations.

Sorted patient samples and cell lines were analysed for functional TRGV(J)C rearrangements using DNA TCRG GeneScan analysis and/or TRGV(J)C qPCR to exclude the presence of contaminating TCRγδ+ lymphocytes or the rare occurrence of cross-lineage TCRγ locus rec36ombinations, which could impede TARP expression. NP indicates no product; neg, negative (no signal). Remaining abbreviations are explained in the legend of Table S1 or in the main text.

4. Supplemental figures

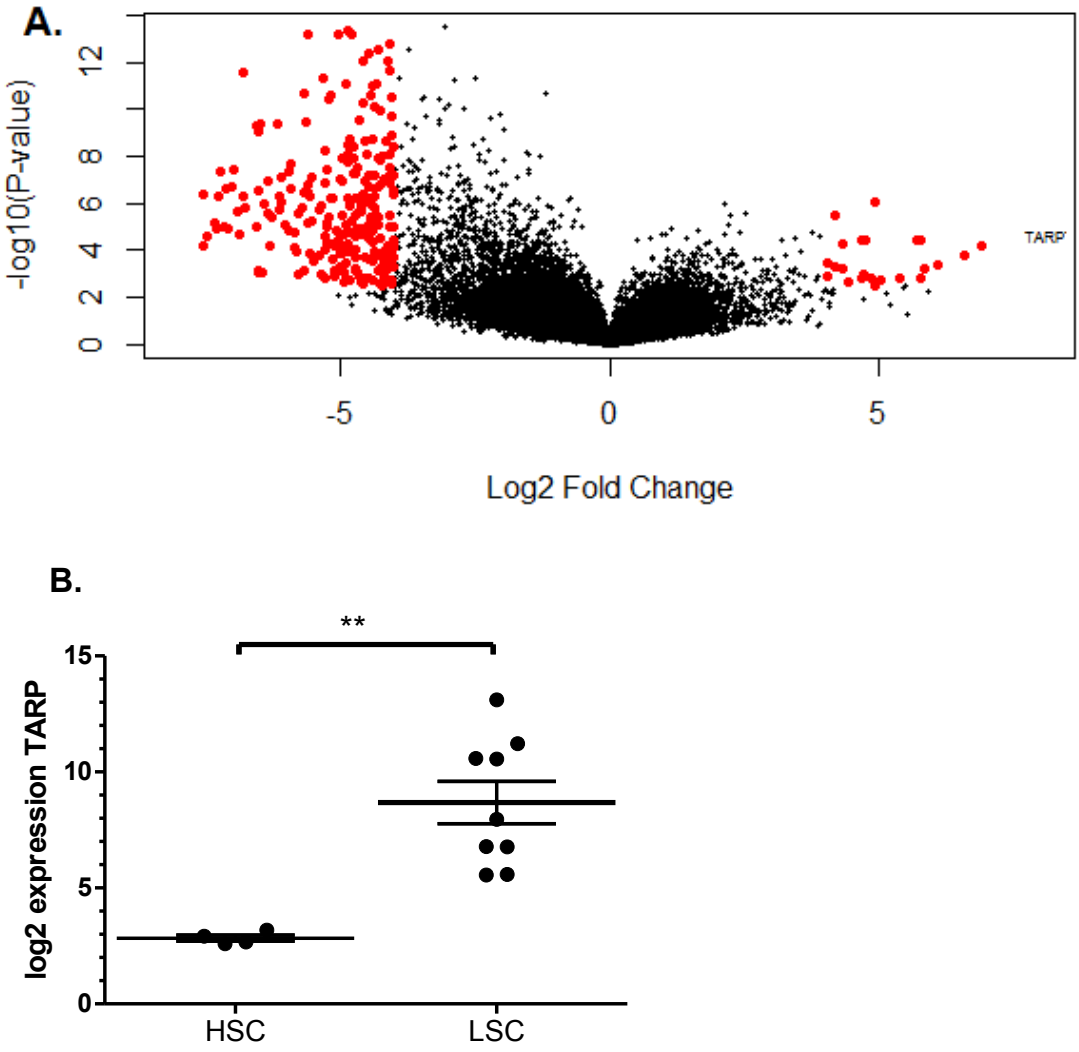


Figure S1. Differential gene expression in leukemic stem cells (LSC) and hematopoietic stem cells (HSC) based on the dataset from Majeti et al.

Array included four probes for the detection of TARP (216920_s_at, 209813_x_at, 215806_x_at and 211144_x_at) within LSCs (n=9) and HSCs (n=4).

(A) Volcano plot showing differentially up- and down regulated genes between LSC and HSC, with 4-fold or higher expression differences (P<0.01) indicated in red. TARP was the most differentially upregulated gene (maximal log2-FC 6.92).

(B) The y-axis represents the TARP mRNA log2 expression values, the x-axis the different sample groups. Horizontal bars indicate means and whiskers are representative for the ±SEM. TARP transcript expression is significantly higher in LSC versus HSC (P<0.01, Mann Whitney U test).

FC indicates fold change. Other abbreviations are explained in the legend of Table 1 or in the main text.

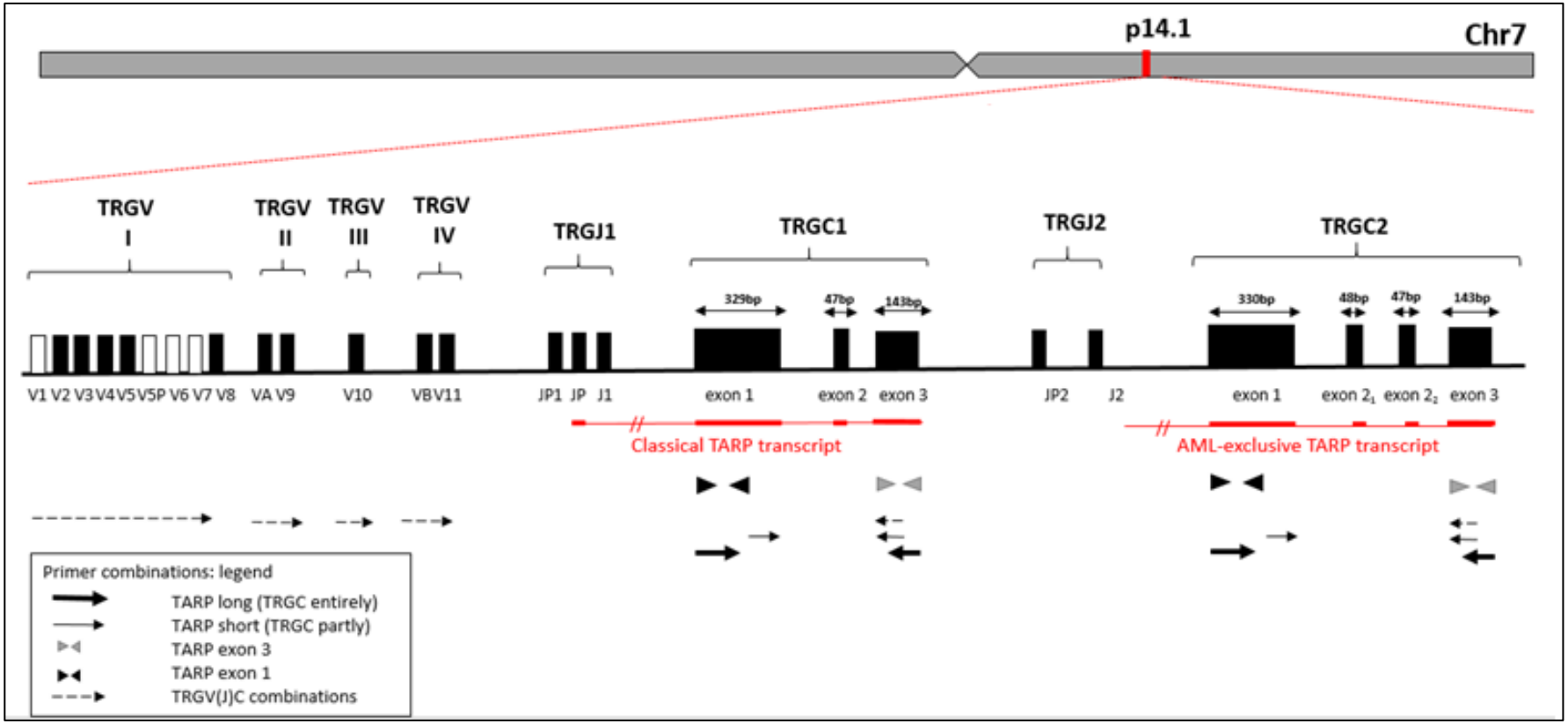
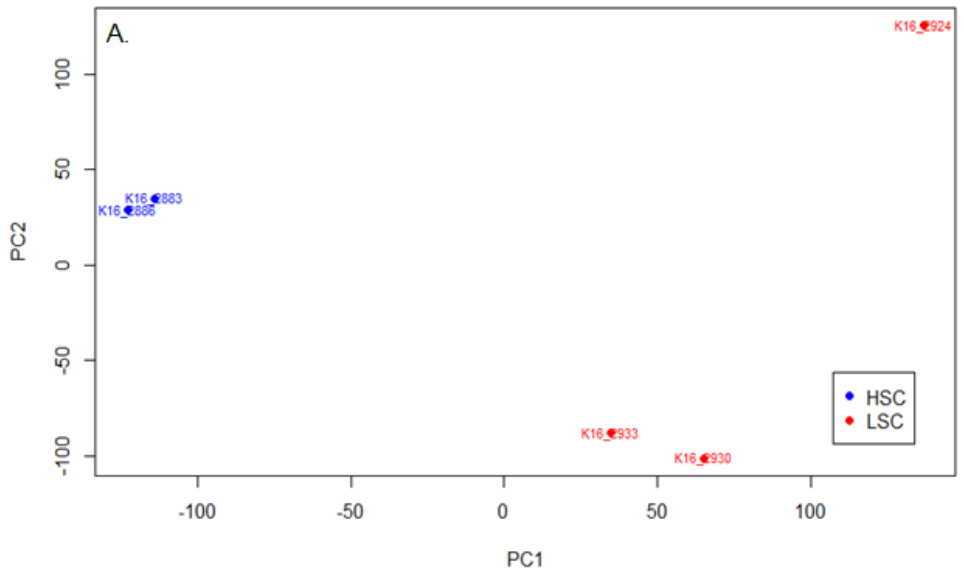
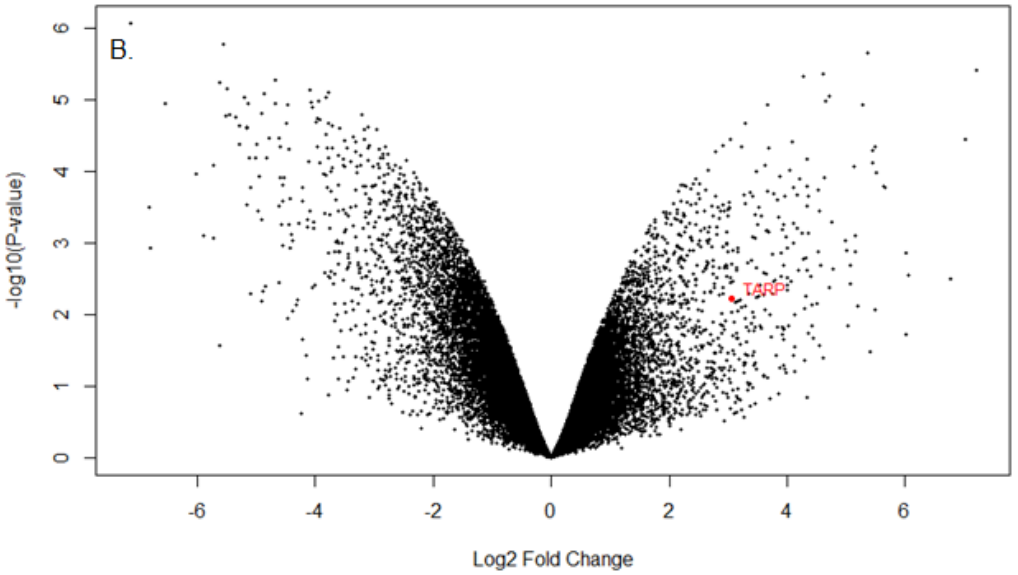


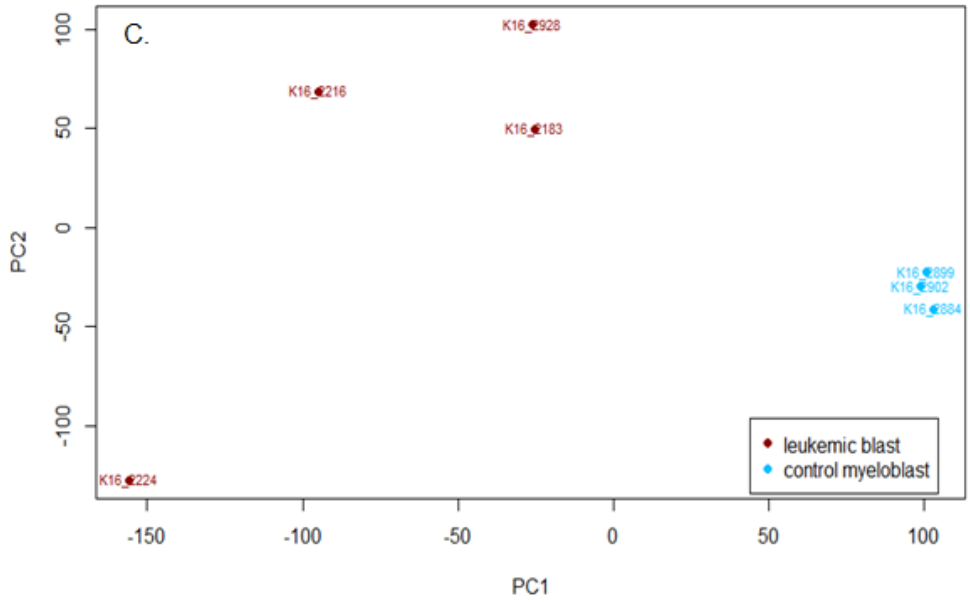
Figure S2. The T-cell receptor γ chain alternate reading frame protein (TARP) at genomic level.

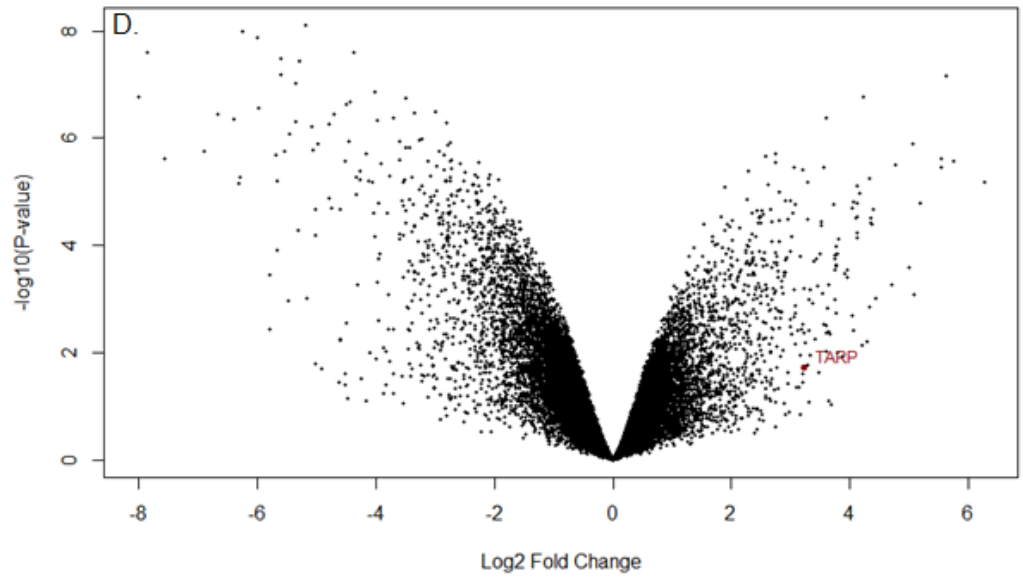
Reference mRNA sequences and annotations were derived from previous work in prostate and breast adenocarcinoma and from the University of California Santa Cruz (UCSC) Genome Browser. The T-cell receptor gamma (TRG) locus, located at 7p15-p14, spans 160 kb and consists out of 12-15 TRG variable (TRGV) genes upstream of a duplicated TRG joining (TRGJ)/TRG constant (TRGC) domain cluster. The TRGV genes are located centromeric with the TRGC2 gene located telomeric in the locus. The first TRGJ/TRGC cluster is composed out of three TRGJ genes (TRGJP1/TRGJP/TRGJ1), each consisting of one exon, and the TRGC1 gene, consisting out of three exons. The second TRGJ/TRGC cluster is separated 16 kb from the first cluster and consists out of two TRGJ genes (TRGJP2/TRGJ2, one exon) and the TRGC2 gene consisting out of four exons. TRGC2 is described to carry a duplicated second exon compared to TRGC1, and both gene segments additionally differ by the presence of single nucleotide variations. The number of base pairs are indicated above each exon of the TRGC locus. Primers used to target part of all TRGC exons (TARP short) are indicated by thin arrows, primers targeting the entire TRGC coding region (TARP long) by thick arrows, and primers targeting solely exon 1 (black) and exon 3 (grey) are indicated by arrow heads. Primers to detect functional TRGV(J)C gene

rearrangements are indicated by arrows with dashed lines. mRNA transcripts for the classical and alternative AML-exclusive TARP transcript are indicated in red.


A.
K16_924
K16_883
K16_888
K16_933
K16_930
HSC
LSC
PC2
PC1
100
50
0
-50
-100
-100
-50
0
50
100


B.
TARP
-log10(P-value)
Log2 Fold Change
0
1
2
3
4
5
6
-6
-4
-2
0
2
4
6


C.
K16_928
K16_216
K16_183
K16_899
K16_902
K16_884
K16_224
leukemic blast
control myeloblast
PC2
PC1
100
50
0
-50
-100
-150
-100
-50
0
50
100


D.
TARP
-log10(P-value)
Log2 Fold Change
0
2
4
6
8
-8
-6
-4
-2
0
2
4
6

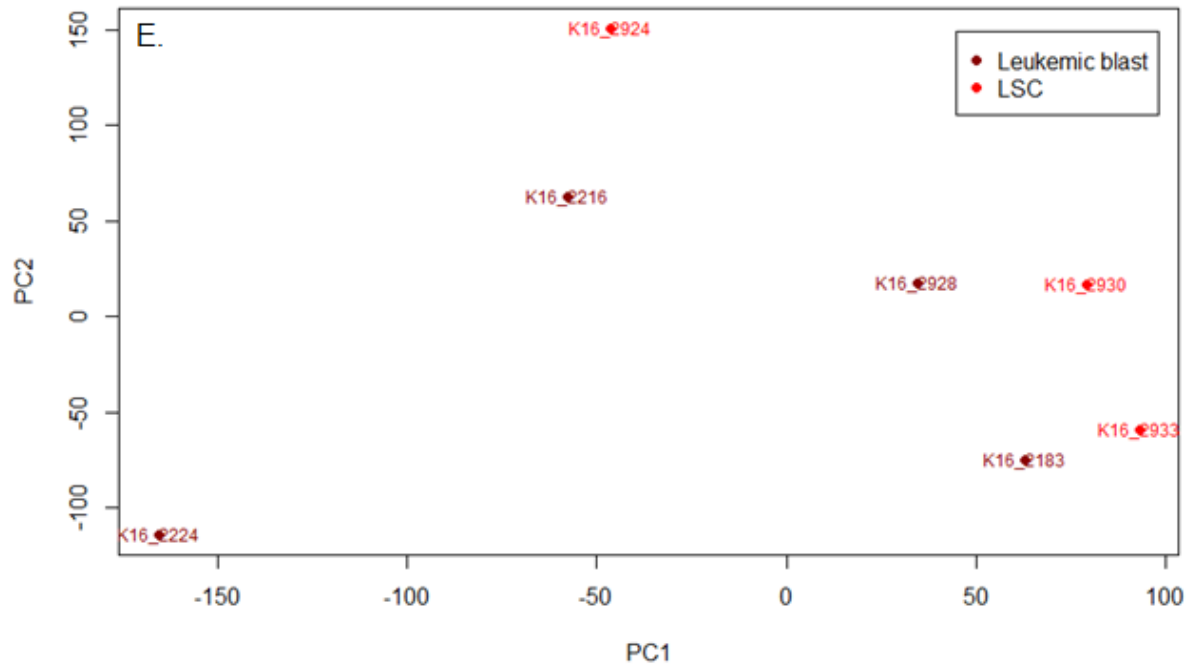


Figure S3. Micro-array profiling of sorted CD34+CD38+ and CD34+CD38- cell fractions from pedAML and cord blood.

CD34+CD38+ (n=4, leukemic blast) and CD34+CD38- (n=3, LSC) cell fractions sorted from four pedAML patients (2 FLT3-ITD, 2 FLT3 WT; Table S1) were used for micro-array profiling, next to sorted CD34+CD38+ (n=3, control myeloblast) and CD34+CD38- (n=2, HSC) cells from cord blood as control populations.

(A) PCA illustrates a clear separation between LSCs (red: K16_2924, K16_2933 and K16_2930) and HSC (blue: K16_2883 and K16_2886).

(B) Volcano plot showing differentially up- and downregulated genes between LSCs and HSCs, with TARP indicated in red (log2-FC 3.06, P<0.01).

(C) PCA illustrates a clear separation between leukemic blasts (dark red: K16_2216, K16_2183, K16_2928, K16_2224) and control myeloblasts (light blue: K16_2884, K16_2899, K16_2902).

(D) Volcano plot showing differentially up- and downregulated genes between leukemic and control myeloblasts, with TARP indicated in dark red (log2-FC 3.22, P<0.05).

(E) PCA illustrating of sorted LSCs (CD34+CD38- (red)) and leukemic blasts (CD34+CD38+ (dark red)) showed a correct clustering on a per patient basis (FLT3 WT pedAML: K16_2183/K16_2933 and K16_2928/K16_2930, FLT3-ITD pedAML: K16_2216/K16_2924). For one FLT3 WT pedAML patient, sorted LSCs were lacking (leukemic myeloblasts K16_2224 indicated in dark red).

PCA indicates Principle Component Analysis. Remaining abbreviations are explained in the main text.

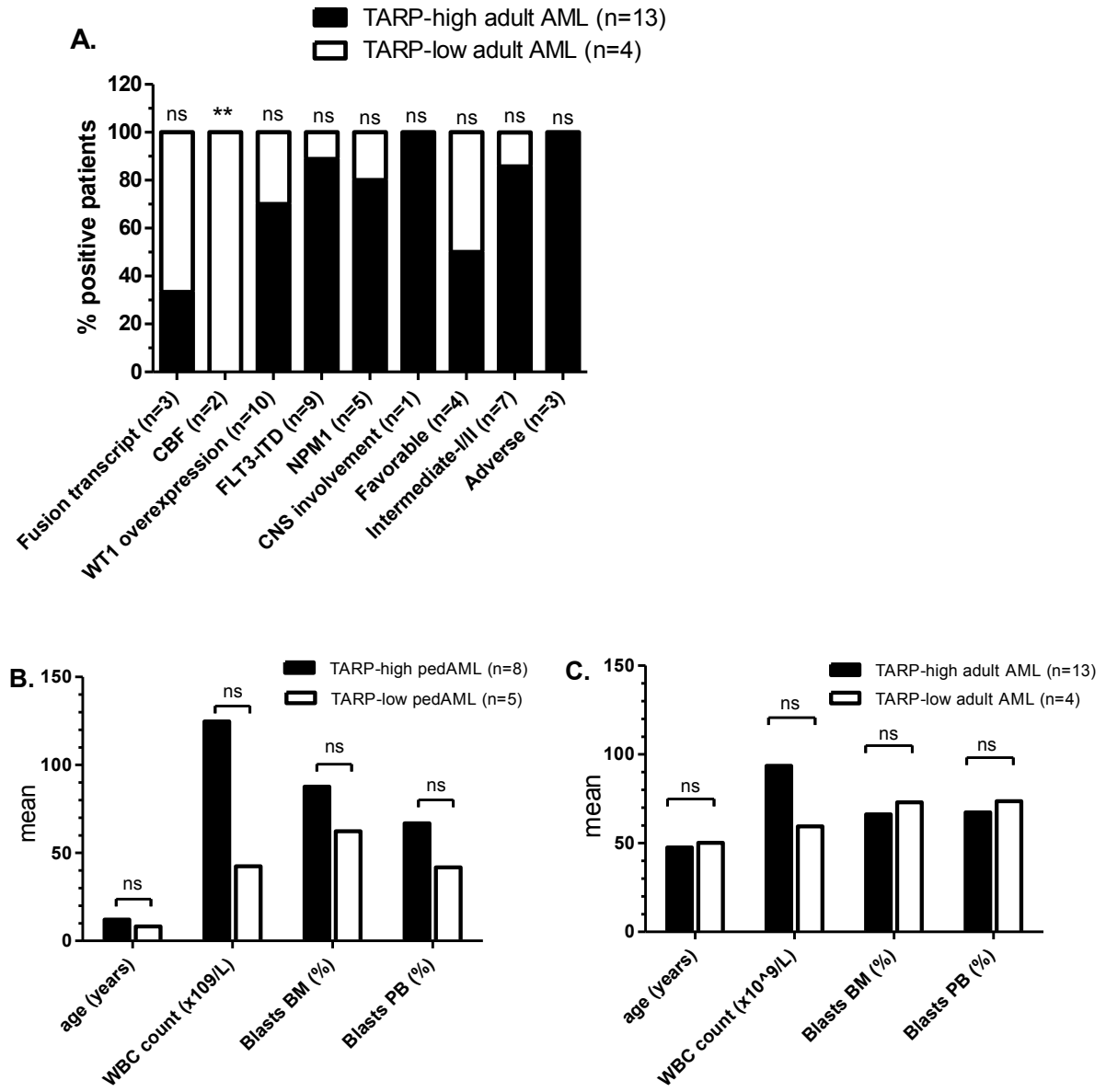
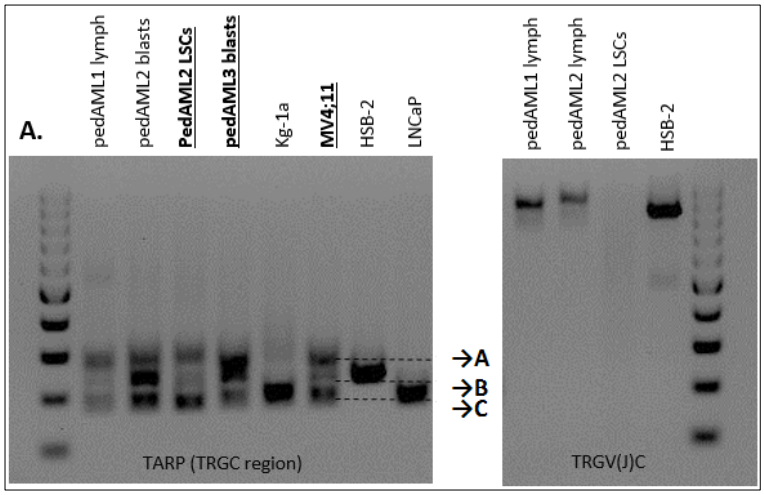


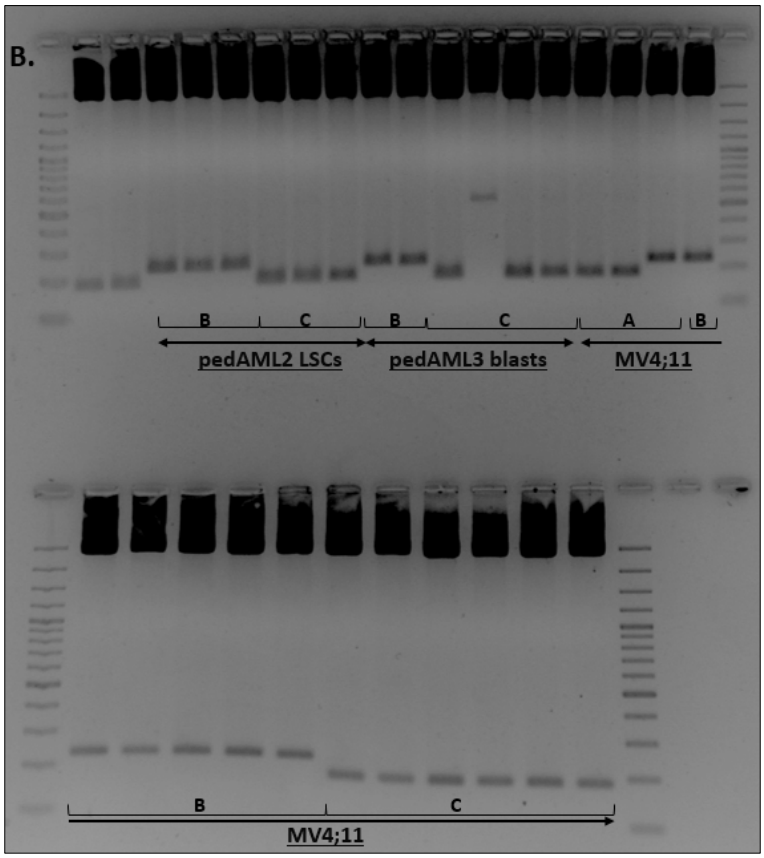
Figure S4. Comparison of characteristics between TARP-high and TARP-low patients.

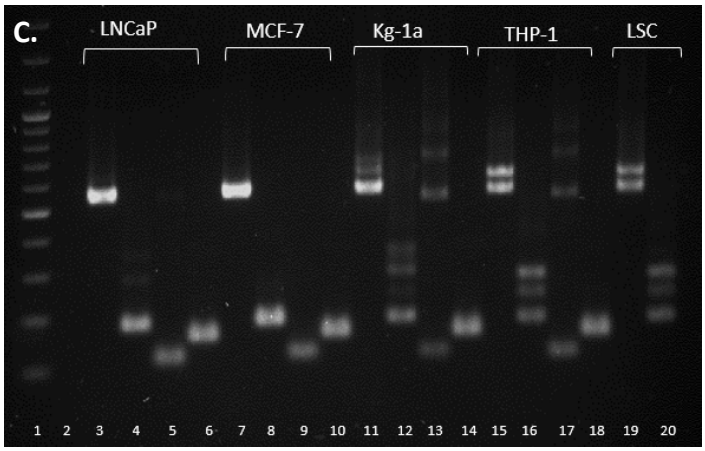
Patients were categorized as TARP-high or TARP-low by qPCR evaluation. Details on the patient characteristics are shown in Table 1. Abbreviations are explained in the previous legends.

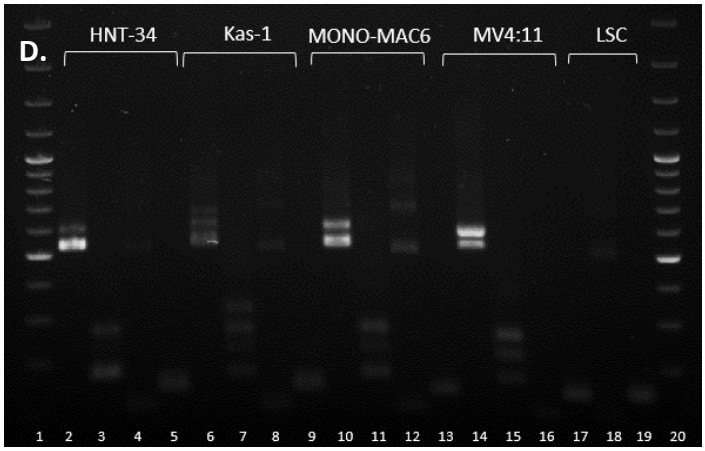
(A) Bars display the percentage of patients harboring the characteristic shown in the x-axis (dichotomous variables), for TARP-high (black, n=13) and TARP-low (white, n=4) adult AML patients. The total number of patients positive for each characteristic is shown between parentheses. Patients without central nerve system (CNS) involvement either had no clinical manifestations or negative lumbar punctures. The number of CBF-leukemia was significantly ($P<0.01$) higher in TARP-low adult AML patients (Chi Square test).

(B-C) Bars display the mean value for the characteristic, shown in the x-axis (continuous variables), calculated for TARP-high (black; pedAML=8 and adult AML=13) and TARP-low (white; pedAML=5 and adult AML=4) patients. No significant differences were detected between adult nor pediatric TARP-high and TARP-low patients (Mann Whitney U test).


A.
pedAML1 lymph
pedAML2 blasts
PedAML2 LSCs
pedAML3 blasts
Kg-1a
MV4;11
HSB-2
LNCaP
pedAML1 lymph
pedAML2 lymph
pedAML2 LSCs
HSB-2
→A
→B
→C
TARP (TRGC region)
TRGV(J)C


B.
B
C
B
C
A
B
pedAML2 LSCs
pedAML3 blasts
MV4;11
B
C
MV4;11


C.
LNCaP
MCF-7
Kg-1a
THP-1
LSC
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20


D.
HNT-34
Kas-1
MONO-MAC6
MV4:11
LSC
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

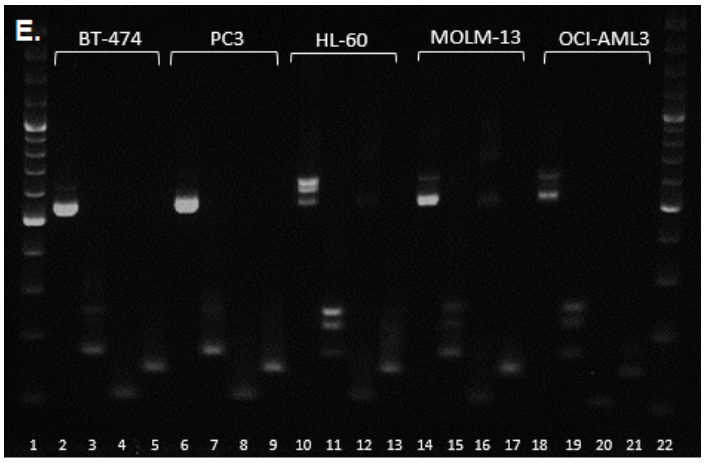

E.
BT-474
PC3
HL-60
MOLM-13
OCI-AML3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Figure S5. TARP transcript analysis.

A GeneRuler 100 bp Plus DNA ladder (range 100 – 3000 bp, 500 and 1000 bp fragment highlighted, ThermoFisher Scientific) was used for size estimation. Origin of the amplicons are indicated at the top or bottom of each lane.

(A) Amplicons obtained by TARP long primers shown for (left to right): pedAML1 lymphocytes, pedAML2 blasts, pedAML2 LSCs, pedAML3 blasts, Kg-1a (AML), MV4;11 (AML), HSB-2 (T-ALL) and LNCaP (prostate adenocarcinoma). PedAML2 en pedAML3 carried FLT3-ITD mutations and were categorized as TARP-high by qPCR, whereas pedAML1 was categorized as TARP-low. Kg-1a, HSB-2 and LNCaP showed single bands, whereas triple bands were present for sorted patients fractions and MV4;11. The smallest fragments (C) were 172 bp in size, middle sized fragments (B) were 48 bp longer (220 bp) and the largest fragments (A) were approximately 250-300 bp. Bands detected for pedAML lymphocytes were proven to be part of functional TCRγ recombinations, comparable to HSB-2 cell line. By contrast, TRGV(J)C rearrangements were excluded for the AML cell line MV4;11 and sorted blasts and LSCs from pedAML patients (Table S4).

(B) DNA was extracted from A, B and C bands for pedAML2 LSCs, pedAML3 blasts and MV4;11 (underlined and bold in (A)), and all purified amplicons were ligated into a pCR®-Blunt vector, followed by transformation, colony picking, miniprep and *Eco*R1 restriction digestion. The digested plasmid of each CFU (2-6 per plasmid), containing either the A, B or C band, was run by gel electrophoresis. Digested products from A fell apart into B or C, and B-fragments were consistently 48 bp longer than C.

(C-E) Two prostate adenocarcinoma cell lines (LNCaP and PC3), two breast adenocarcinoma cell lines (BT-474 and MCF-7), nine AML cell lines (MOLM-13, MV4;11, Kg-1a, THP-1, HNT-34, Kas-1, MONO-MAC6, HL-60 and OCI-AML3) and pedAML2 LSCs were targeted for amplification by four different primer pairs e.g. TARP short, TARP long, TARP exon 1 and TARP exon 3 (genomic location primers shown in Fig. S2). Amplicons are shown per cell line in this respective order, adjacent to each other, except for pedAML LSC amplicons which were divided over two gels (first two amplicons C, last two amplicons in D). Band intensities agreed with expression levels determined by qPCR (Fig. 1F).

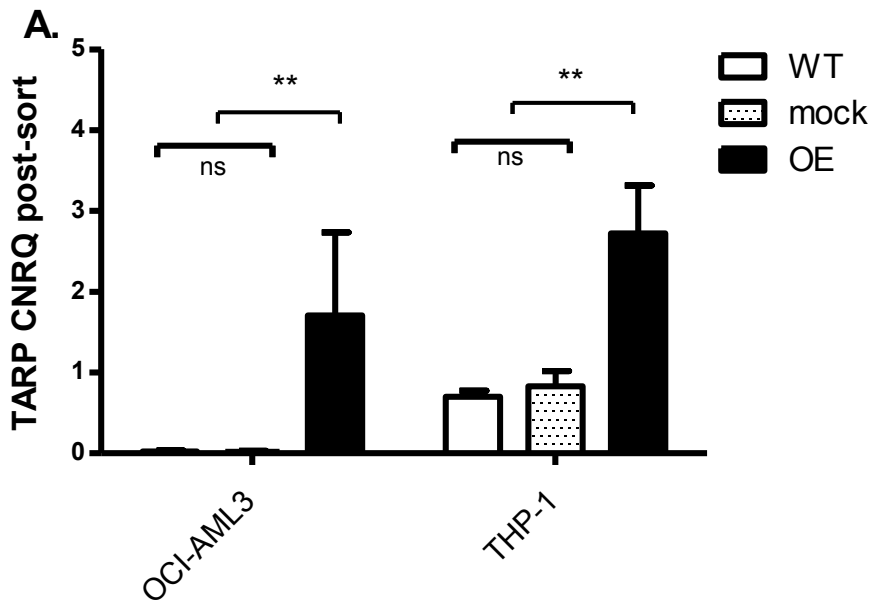
CFU indicates colony-forming-units; Kas-1, Kasumi-1. Remaining abbreviations are explained in the previous legends or in the main text.

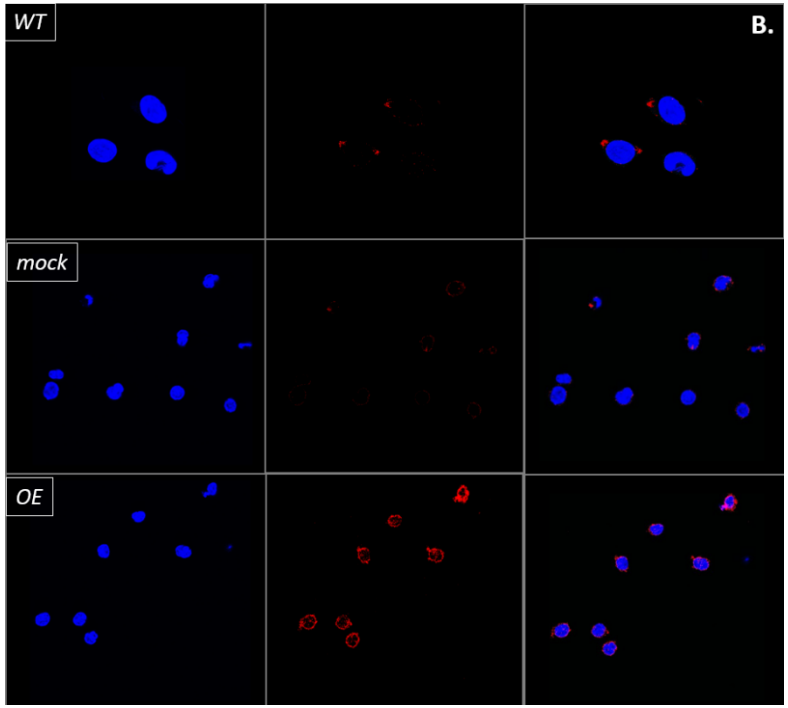
Figure S6. Differential TARP transcript and protein expression levels observed in transgenic OE, mock and WT OCI-AML3 and THP-1 cell lines.

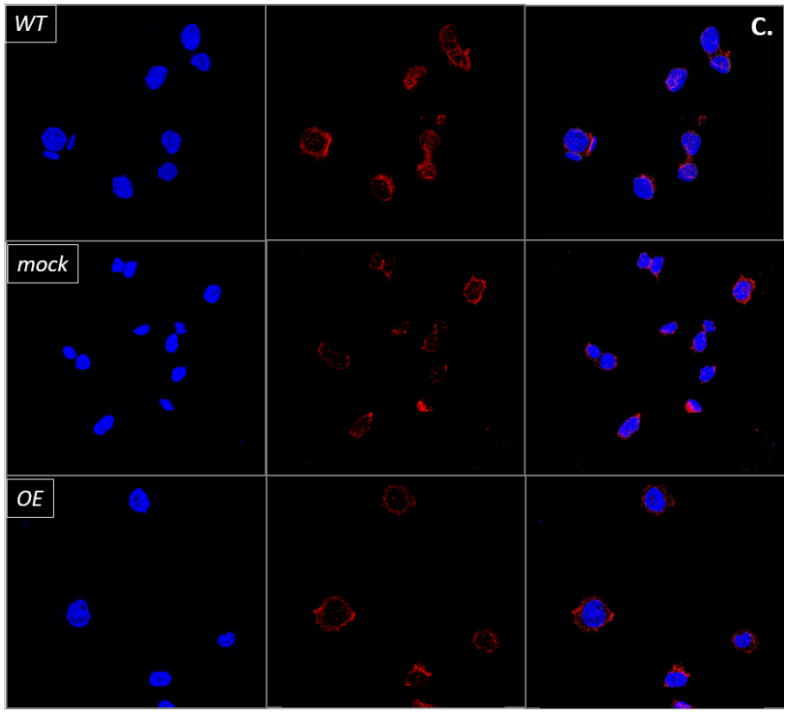
(A) Transgenic TARP OE cell lines showed significantly higher expression levels compared to the mock and WT parental lines (P<0.01, Mann-Whitney U test). TARP expression was based on biological triplicates, and error bars indicate ±SEM.

(B-C) Comparison of TARP protein levels between WT (top), mock (middle) and transgenic OE (bottom) cell lines generated for OCI-AML3 (B) and THP-1 (C). The first column illustrates only DAPI counterstaining, the second column only TARP staining, and the third column merged images. TARP protein levels were quite comparable between the OE, TC and WT cell lines for THP-1. By contrast, the transgenic OCI-AML3 OE cell line showed an increased TARP protein expression compared to the mock and WT cell line.

OE indicates overexpression; WT: wild-type. Remaining abbreviations are explained in the previous legends or in the main text.







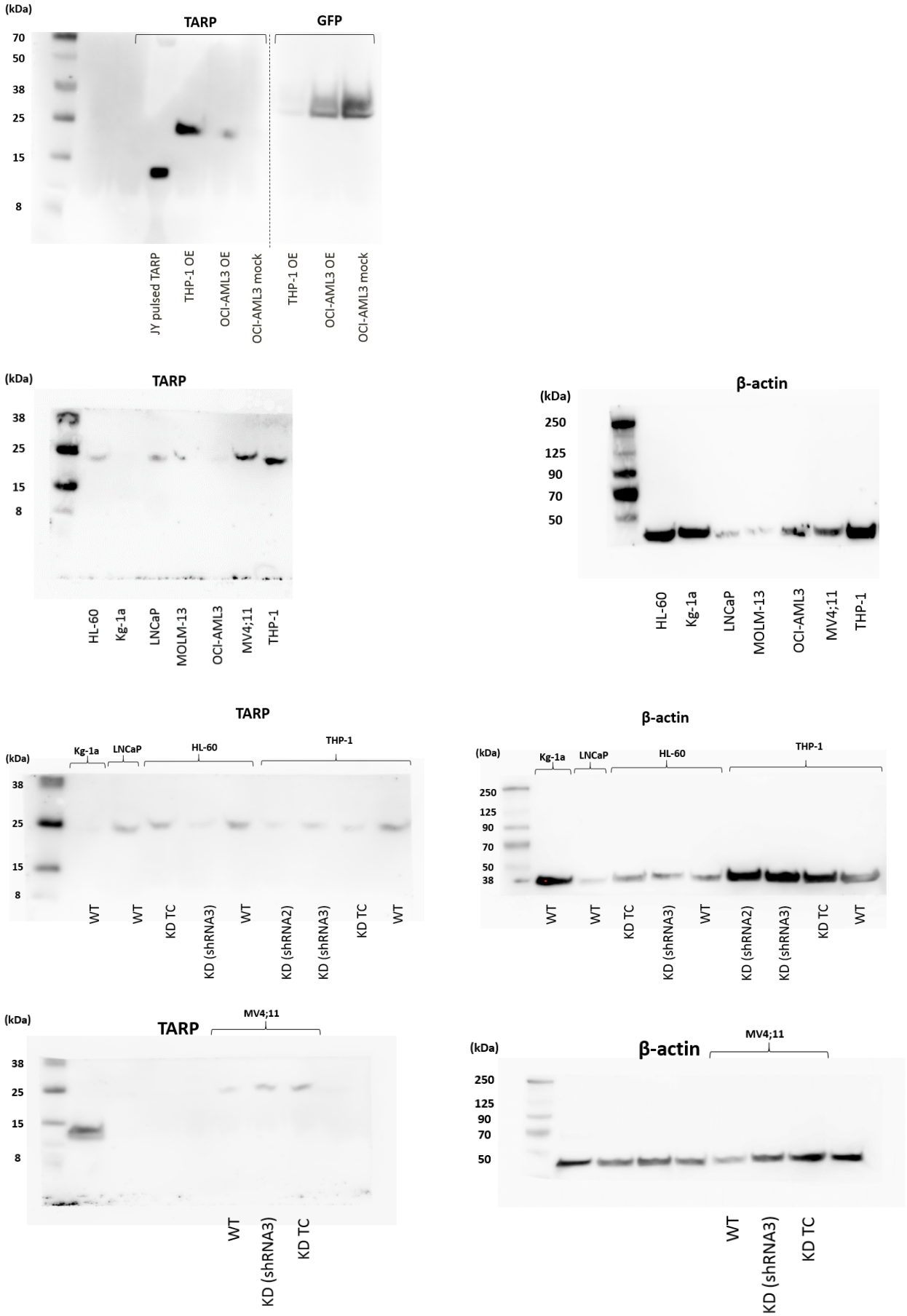


Figure S7. Whole-blot Western blot images.

Overview of the whole-blot images, including a pre-stained ladder (WesternSure), which were used to generate Fig. 2 in the main document. B-C, D-E and F-G derived from the same blot and were cut just above the 38 kDa ladder fragment to allow simultaneous TARP and β-actin staining and avoid reprobing.

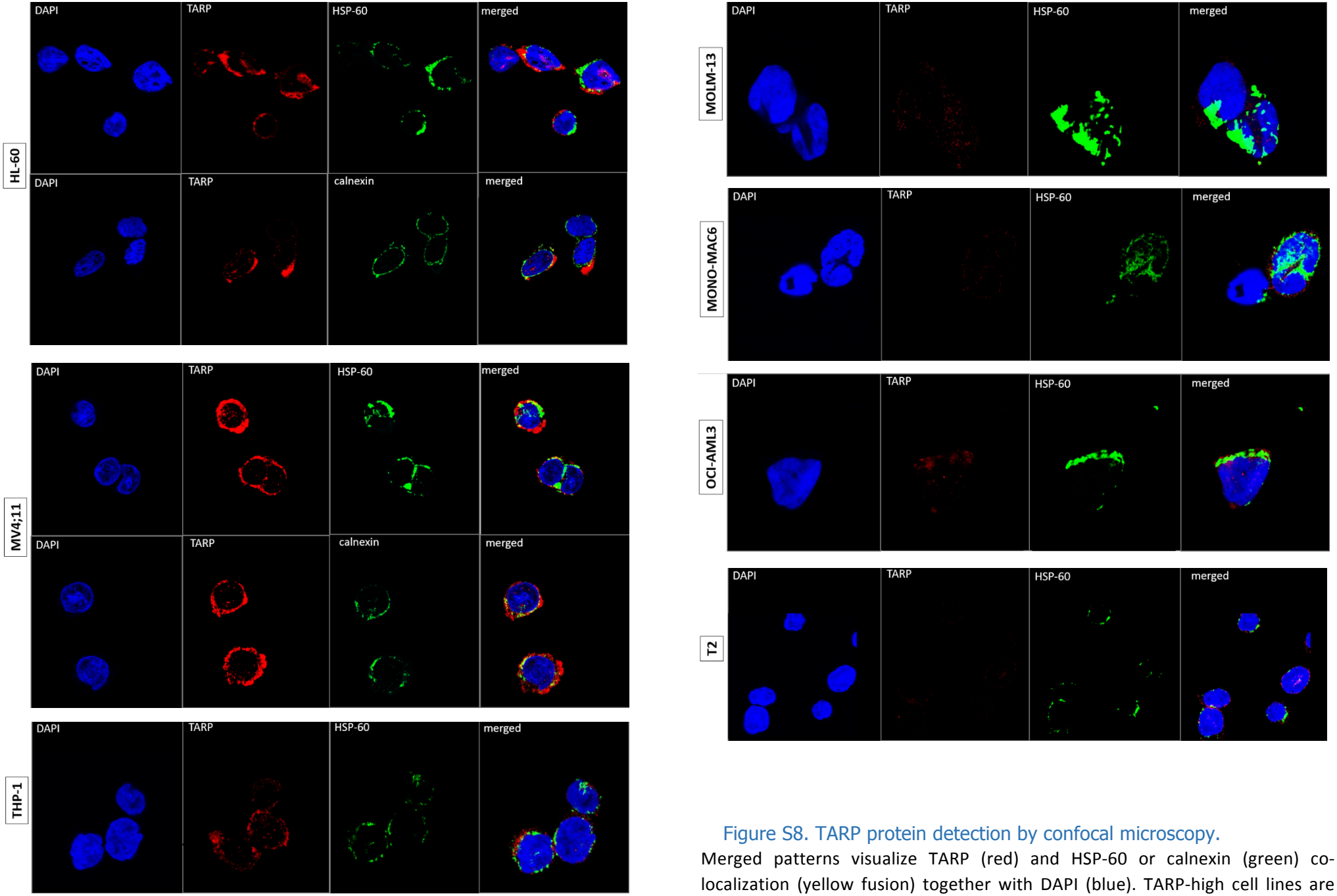


Figure S8. TARP protein detection by confocal microscopy.

Merged patterns visualize TARP (red) and HSP-60 or calnexin (green) co-localization (yellow fusion) together with DAPI (blue). TARP-high cell lines are

shown on the left (HL-60, MV4;11 and THP-1), TARP-low cell lines (MOLM-13, MONO-MAC6, OCI-AML3 and T2) on the right. Calnexin colocalization was unsuccessful for THP-1 and not evaluated for TARP-low cell lines.

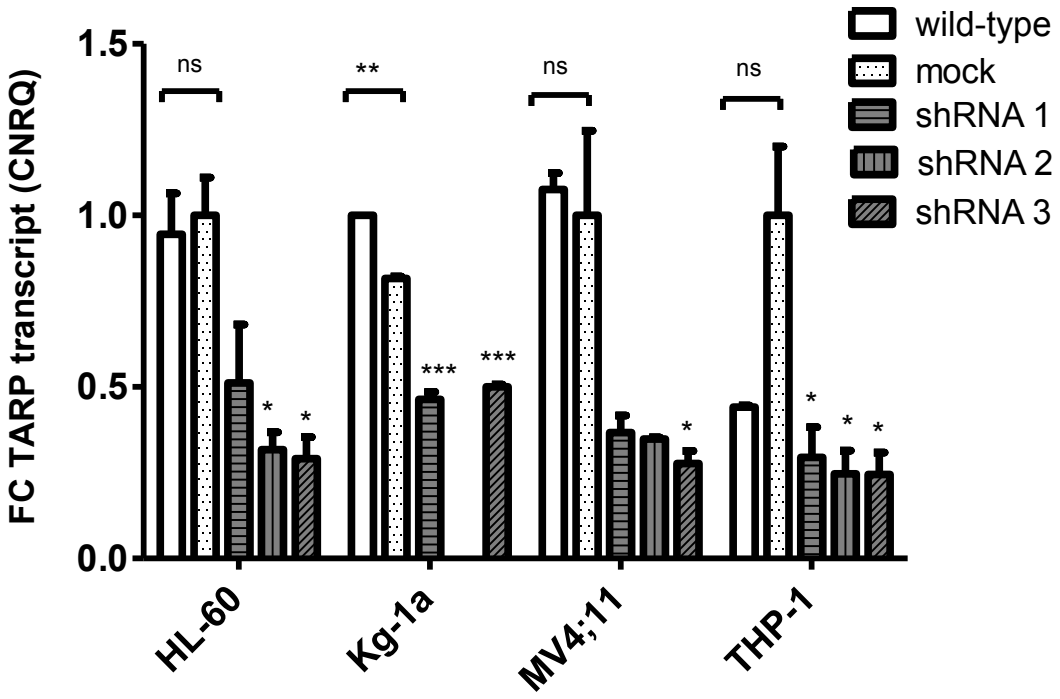


Figure S9. Differential TARP transcript expressions between KD, mock and wild-type cell lines.
Knockdown (shRNA 1, 2, 3) and mock cell lines were generated for HL-60, Kg-1a, MV4;11 and THP-1. Transcript expression levels (CNRQ) are based on biological duplicates and expressed as fold change (FC) versus mock (HL-60, MV4;11 and THP-1) or WT (Kg-1a) cell line, with error bars indicative for ±SEM. Due to low viability during culture, Kg-1a transduced with shRNA 2 could not be evaluated. No significant differences were observed between TC and WT cell lines, although THP-1 TC was marginally upregulated, except for Kg-1a ($P<0.01$, Mann-Whitney U test). Significant downregulation was therefore calculated between each KD cell line and the respective TC (HL-60, MV4;11, THP-1), or the WT cell line in case of Kg-1a, using One-way ANOVA with Tukey's Multiple Comparison post-test. If significant, one ($P<0.05$), two ($P<0.01$) or three ($P<0.001$) asterisks are indicated above the respective chart.

KD indicates knockdown. Remaining abbreviations are explained in the previous legends or in the main text.

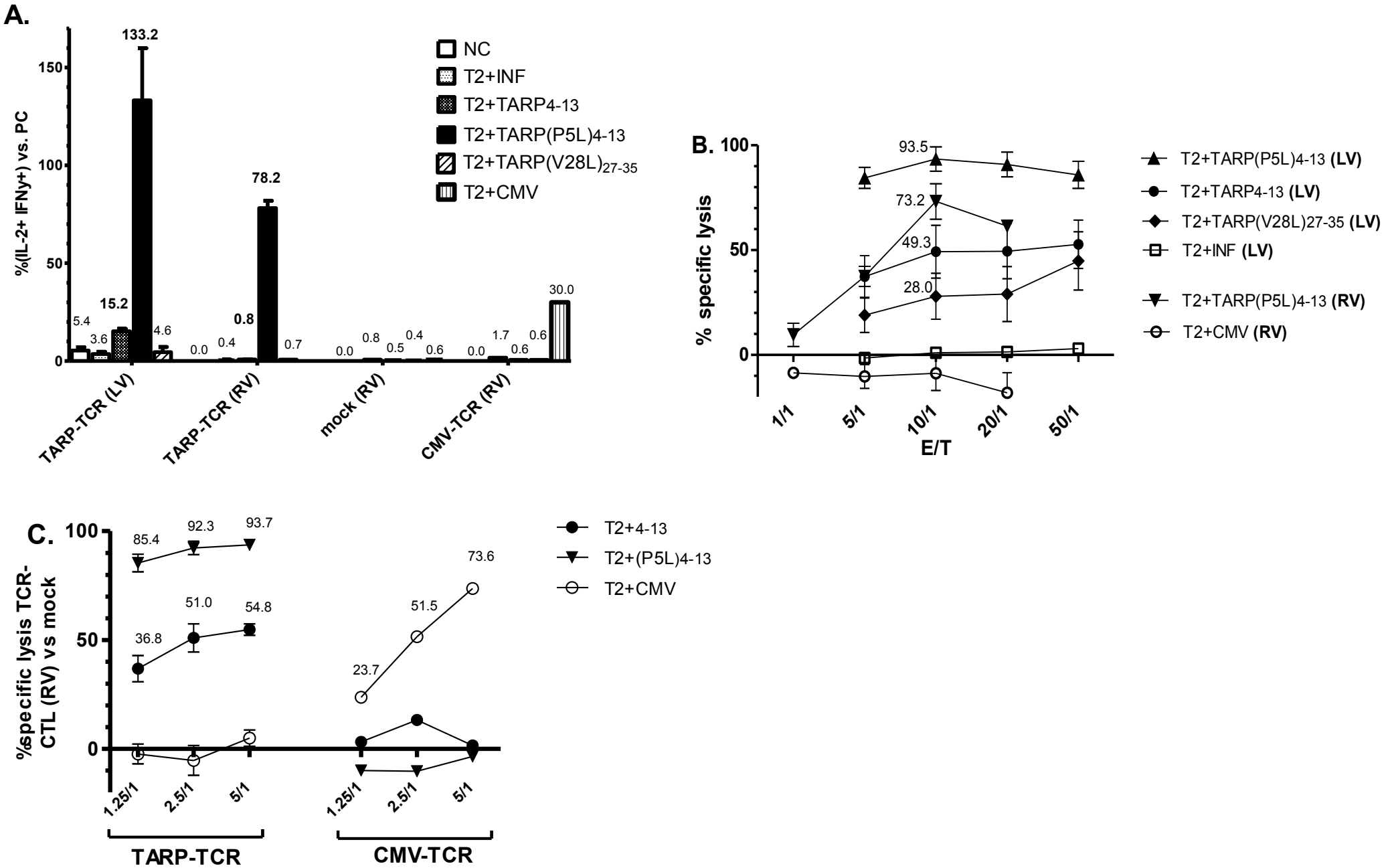

A.
%(IL-2+ IFNγ+) vs. PC
150
100
50
0
133.2
78.2
30.0
15.2
0.8
5.4
3.6
4.6
0.0
0.4
0.7
0.0
0.8
0.5
0.4
0.6
0.0
1.7
0.6
0.6
TARP-TCR (LV)
TARP-TCR (RV)
mock (RV)
CMV-TCR (RV)
NC
T2+INF
T2+TARP4-13
T2+TARP(P5L)4-13
T2+TARP(V28L)27-35
T2+CMV
B.
% specific lysis
100
50
0
93.5
73.2
49.3
28.0
1/1
5/1
10/1
20/1
50/1
E/T
T2+TARP(P5L)4-13 (LV)
T2+TARP4-13 (LV)
T2+TARP(V28L)27-35 (LV)
T2+INF (LV)
T2+TARP(P5L)4-13 (RV)
T2+CMV (RV)
C.
%specific lysis TCR-CTL (RV) vs mock
100
50
0
85.4
92.3
93.7
51.0
54.8
36.8
23.7
51.5
73.6
1.25/1
2.5/1
5/1
TARP-TCR
CMV-TCR
T2+4-13
T2+(P5L)4-13
T2+CMV

Figure S10. Functional evaluation of TCR-transgenic CTLs towards pulsed T2 cells.

An overview of the peptides used for pulsing T2 cells is shown in Table S3. Error bars indicate ±SEM based on two, four or six biological replicates, depending on the experiment, and mean values are shown above error bars if applicable.

(A) Feeder-expanded LV and RV TARP-TCR CTLs were incubated with pulsed T2 cells. Cytokine release was calculated based on IFN-γ/IL-2 double positive cell populations within the CD3+/CD8+ compartment, and expressed relatively to the positive control. TARP-TCR CTLs exerted a peptide-specific IFN-γ and IL-2 production, reacting stronger against the TARP(P5L)$_{4\text{-}13}$ than the cognate TARP$_{4\text{-}13}$ peptide. RV transduced TARP-TCR CTLs appeared to be less responsive than LV transduced CTLs, though remained specific, as mock CTLs did not respond and CMV-TCR CTLs only reacted against the CMV peptide.

(B) Chromium^{51} release assay evaluated killing by LV TARP-TCR CTLs (E/T ratio 5/1, 10/1, 20/1, 50/1: upper part of legend) and by RV TARP-TCR CTLs (E/T ratio 1/1, 5/1, 10/1, 20/1: bottom part of legend). TARP(P5L)$_{4\text{-}13}$ pulsed T2 cells were more efficiently lysed than TARP$_{4\text{-}13}$ pulsed T2 cells. For both targets, lysis started from an E/T ratio 5/1 and peaked at E/T 10/1. Killing evaluated by RV transduced CTLs was lower compared to LV transduced CTLs (e.g. for TARP(P5L)$_{4\text{-}13}$: 93.5% vs. 73.2% at ratio 10/1).

(C) FCM-based cytotoxicity assay (24-h) illustrating cytotoxic killing of TARP-related peptides (black symbols) and TARP-unrelated peptides (white symbols). Llysis started from E/T 1.25/1, with again higher killing towards TARP(P5L)$_{4\text{-}13}$ versus TARP$_{4\text{-}13}$ (mean 90.5% vs. 47.5%), while non-TARP related peptides remained unaffected.

CTL indicates cytotoxic T-cells; FCM, flow cytometry; IFN-γ, interferon gamma; IL-2, interleukin-2; LV, lentiviral; RV, retroviral. Remaining abbreviations are explained in the previous legends or in the main text.

5. Supplemental references

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