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

Barbara Depreter,[1,2] Karin E. Weening,[2,3] Karl Vandepoele,[2,4] Magnus Essand,[5] Barbara De Moerloose,[1,2,6] Maria Themeli,[7] Jacqueline Cloos,[7] Diana Hanekamp,[7] Ine Moors,[8] Inge D'hont,[6] Barbara Denys,[2,4] Anne Uyttebroeck,[9] An Van Damme,[10] Laurence Dedeken,[11] Sylvia Snauwaert,[12] Glenn Goetgeluk,[3] Stijn De Munter,[2,3] Tessa Kerre,[2,8] Bart Vandekerckhove,[2,3] Tim Lammens[1,2*] and Jan Philippé[2,3,4*]

[1]Department of Internal Medicine and Pediatrics, Ghent University, Ghent, Belgium; [2]Cancer Research Institute Ghent, Ghent University, Ghent, Belgium; [3]Department of Diagnostic Sciences, Ghent University, Ghent, Belgium; [4]Department of Laboratory Medicine, Ghent University Hospital, Ghent, Belgium; [5]Science for Life Laboratory, Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden; [6]Department of Pediatric Hematology-Oncology and Stem Cell Transplantation, Ghent University Hospital, Ghent, Belgium; [7]Department of Hematology, VU University Medical Center, Amsterdam, the Netherlands; [8]Department of Hematology, Ghent University Hospital, Ghent, Belgium; [9]Department of Pediatrics, University Hospital Gasthuisberg, Louvain, Belgium; [10]Department of Pediatric Hematology Oncology, University Hospital Saint-Luc, Brussels, Belgium; [11]Department of Pediatric Hematology Oncology, Queen Fabiola Children's University Hospital, Brussels, Belgium and [12]Department of Hematology, AZ Sint-Jan Hospital Bruges, Bruges, Belgium

*JP and TL contributed equally to this work as co-senior authors.

Correspondence:

TIM LAMMENS
tim.lammens@ugent.be

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/105/5/1306

ABSTRACT

Immunotherapeutic strategies targeting the rare leukemic stem cell compartment might provide salvage to the high relapse rates currently observed in acute myeloid leukemia (AML). We applied gene expression profiling for comparison of leukemic blasts and leukemic stem cells with their normal counterparts. Here, we show that the T-cell receptor γ chain alternate reading frame protein (TARP) is over-expressed in *de novo* pediatric (n=13) and adult (n=17) AML sorted leukemic stem cells and blasts compared to hematopoietic stem cells and normal myeloblasts (15 healthy controls). Moreover, TARP expression was significantly associated with a fms-like tyrosine kinase receptor-3 internal tandem duplication in pediatric AML. TARP overexpression was confirmed in AML cell lines (n=9), and was found to be absent in B-cell acute lymphocytic leukemia (n=5) and chronic myeloid leukemia (n=1). Sequencing revealed that both a classical TARP transcript, as described in breast and prostate adenocarcinoma, and an AML-specific alternative TARP transcript, were present. Protein expression levels mostly matched transcript levels. TARP was shown to reside in the cytoplasmic compartment and showed sporadic endoplasmic reticulum co-localization. TARP-T-cell receptor engineered cytotoxic T-cells *in vitro* killed AML cell lines and patient leukemic cells co-expressing TARP and HLA-A*0201. In conclusion, TARP qualifies as a relevant target for immunotherapeutic T-cell therapy in AML.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous hematologic malignancy, accounting for 80% of adult[1-4] and 20% of pediatric[5-7] leukemia. Despite initial clinical remission rates of 60-90%,[2,5,6] patients exhibit a high relapse risk and therapy-related mortality, resulting in a 5-year overall survival of 30% in adult AML[1,3] and 65-70% in pediatric AML (pedAML).[5,8] Especially the prognosis of patients with fms-like tyrosine kinase receptor-3 internal tandem duplications (*FLT3*-ITD) remains extremely poor.[2,8,9] The high relapse rate is thought to arise from a chemotherapy-resistant cell fraction with unlimited self-renewal capacities,

denominated as leukemic stem cells (LSC).[4,10-14] In CD34+ AML, stem cell characteristics were shown to be present in all four CD34/CD38 phenotypic compartments, though the CD34+CD38− fraction was the most LSC-enriched.[15] Moreover, a high LSC load at diagnosis was shown to be a significant adverse prognostic factor.[16-19] Unfortunately, current chemotherapeutic regimens were shown to perform inadequate towards LSC eradication[14] and they induce important toxicity.[5,6,20] Also hematopoietic stem cell transplantation, performed in high-risk (HR) patients or as salvage therapy, carries a high mortality and morbidity risk,[2,5] highlighting the need for alternative treatments. Thus, identifying LSC aberrations is crucial to tackle the high relapse rate and to develop therapeutic targeting strategies for LSC elimination, while ensuring salvage of normal hematopoietic stem cells (HSC).

Targeted therapy has led to remarkable progress in the survival rates of multiple cancers. The introduction of tyrosine kinase inhibitors in the treatment of chronic myeloid leukemia (CML) accomplished a major breakthrough, and CD19-directed chimeric antigen receptor (CAR) therapy has led to an enormous improvement in survival in relapsed/refractory pediatric ALL.[21,22] These successes paved the way for the exploration of the clinical applicability of targeting antibodies and CAR- or T-cell receptor (TCR)-transgenic cytotoxic T cells (CTL) in AML.[2,23-28] Although an increasing number of LSC-specific membrane markers have been identified over recent years,[18,23,29,30] only a few reports address the molecular abnormalities of LSC compared to HSC,[15,31-37] especially in pedAML.

Here, we identified the T-cell receptor (TCR)γ chain alternate reading frame protein (TARP) as an AML-specific target, expressed in the LSC and blasts of pediatric and adult AML, while absent in their normal counterparts. *TARP* transcript expression was associated with *FLT3*-ITD in pedAML. In addition, we provide *in vitro* evidence that TARP may serve as a novel immunotherapeutic target in AML for TARP-TCR engineered CTL.

Methods

Patients

We retrospectively selected diagnostic material from 13 pedAML and 17 adult AML patients based on the sample availability, LSC load, CD34 positivity, *FLT3* mutational status, and HLA-status (Table 1 and *Online Supplementary Table S1*). At diagnosis, mononuclear cells (MNC) were isolated from bone marrow (BM) or peripheral blood (PB) by Ficoll density gradient (Axisshield) and cryopreserved in 90% fetal calf serum (FCS) and 10% dimethylsulfoxide (DMSO). Samples were thawed, followed by 30 minutes (min) incubation at room temperature (RT) in 20 mL RPMI with 20% FCS, 200 μL DNase I (1 mg/mL, grade II bovine pancreas), and 200 μL MgCl2 (1 M) (Sigma-Aldrich). After incubation, cells were spinoculated (10 min, 400 rpm) and washed once more with RPMI/20% FCS.

In addition, we prospectively collected material from 15 healthy subjects. Normal bone marrow (NBM, n=6) was collected from posterior iliac crest of pediatric patients (4-18 years) undergoing scoliosis surgery. Umbilical cord blood (CB, n=7) was obtained after normal vaginal deliveries at full term. Mobilized peripheral blood stem cells (mPBSC, n=2) were collected by apheresis of adult donors pre-allotransplant. All patients or their guardians gave their informed consent and approval was obtained by the ethical committee, in accordance with the Declaration of Helsinki. Buffy coats from donors were obtained from the Red Cross (Mechelen, Belgium) and used for CTL isolation and the preparation of feeder cell medium.

Flow cytometry analysis and cell sorting

Cell pellets were surface stained (*Online Supplementary Table S2*), followed by 20 min incubation at 4°C and washing with PBS+2% BSA. For cell-sorting, labeled cells were resuspended in medium and sorted on a FACSAria III with red, blue, and violet lasers (BD Biosciences). For flow cytometry (FCM) analysis, cells were resuspended in PBS+2% BSA and analyzed on a LSR II or a FACSCanto II, equipped with four or three solid-state lasers, respectively (both BD Biosciences). All scatters were devoid of doublets based on FSC-H/FSC-A, and propidium iodide (PI) was used to exclude dead cells. Sorting strategies are described in *Online Supplementary Data 2.2*. Regarding FCM-based cytotoxicity and cytokine assays (*Online Supplementary Data 2.9*), living cells were selected using a LIVE/DEAD staining (1:10000 dilution, ThermoFisher Scientific) instead of PI. Target cells were stained with a Violet CellTrace™ (VT) Cell Proliferation Kit (5 mM, 1:10000 dilution, ThermoFisher Scientific) prior to incubation with TCR-engineered CTL. After incubation and before surface staining, Flow-Count™ Fluorospheres (1:20 diluted, Beckman Coulter) were added to each well to enable target quantification (measurement of minimum 1000 Fluorospheres/well).

Transcript expression

Details on micro-array profiling, RNA isolation, cDNA synthesis, quantitative polymerase chain reaction (qPCR) conditions and primers can be found in *Online Supplementary Data 2.3, 2.4, 2.5* and *Online Supplementary Table S3*. qPCR data analysis was performed according to state-of-the-art methods.[38,39] Relative quantity (RQ) values were normalized against housekeeping genes *GAPD, HPRT1* and *TBP*. For *TARP* expression, normalized relative quantities were calibrated (calibrated normalized relative quantity, CNRQ) *versus* a single calibrator to allow interrun comparison. For the investigation of the subcellular localization of TARP, delta (d) Ct between cytoplasmic and nuclear compartments were calculated and compared to *MALAT1* and *TBP* expression. Functional TCRG gene rearrangements were excluded if sufficient material remained using DNA TCRG GeneScan analysis40 and/or TRGV(J)C qPCR (*Online Supplementary Table S4*).

Protein detection

Details on western blotting and confocal microscopy are provided in *Online Supplementary Data 2.6*.

Viral transduction of acute myeloid leukemia cell lines and generation of T-cell receptor-transgenic cytotoxic T cells

All transfer and helper plasmids used, and procedures for transformation, plasmid isolation, transfection and transduction are described in *Online Supplementary Data 2.7 and 2.8*.

Six AML cell lines (HL-60, Kg-1a, MOLM-13, HL-60-Luc, MOLM-13-Luc and MV4;11-Luc) were transduced with HLA-A*0201 MHC-I encoding retrovirus, hereafter defined as A2+. Transgenic TARP overexpression (OE) cell lines were generated for OCI-AML3 and THP-1, next to mock controls. TARP was knocked down in four TARP-high AML cell lines (HL-60, Kg-1a, MV4;11 and THP-1) using three different shRNA, next to mock controls.

TARP-TCR engineered CTL were generated by transduction with lentiviral (LV) or retroviral (RV) particles encoding a TCRA8-T2A-TCRB12 sequence directed against the HLA-A*0201-restrict-

Table 1. Characteristics of *de novo* acute myeloid leukemia (AML) patients used for sorting CD34⁺CD38⁺ and CD34⁺CD38⁻ cell fractions and qualitative polymerase chain reaction evaluation.

	Pediatric AML (n=13) Median (Range)		Adult AML (n=17) Median (Range)	
Age, years	10 (2-16)		48 (20 - 76)	
WBC count, x 10⁹/L	66 (2.7-336)		15 (6-274)†	
Morphological blast count				
BM, %	81 (34-96)		77 (28-90)†	
PB, %	67 (1-95)		73 (7-93)‖	
	N	**%**	**N**	**%**
Gender				
F	7	53.8%	9	52.9%
M	6	46.2%	8	47.1%
Sample				
BM	8	61.5%	11	64.7%
PB	5	38.5%	6	35.3%
CD34 positivity	13	100.0%	15	88.2%
Fusion transcript	6	46.2%	3*	18.8%
CBF leukemia	4	30.8%	2	11.8%
WT1 overexpression	10	76.9%	10‡	71.4%
Mutation status				
NPM1	0	0.0%	5‡	35.7%
FLT3-ITD	8	61.5%	9†	60.0%
Risk classification				
SR	7	53.8%	Favorable	423.5%
HR	5	38.5%	Intermediate-I/II	741.2%
Unknown	1	7.7%	Adverse	317.6%
			Unknown	317.6%

Pediatric acute myeloid leukemia (pedAML) patients were diagnosed in Belgium and treated according to the DB AML-01 (n=9, 69%) or NOPHO-DBH AML 2012 (n=4, 31%) protocol. Pediatric patients were risk stratified as previously published⁸ and categorized according to the French-American-British (FAB) classification into M0 (n=1), M1 (n=1), M2 (n=4), M3 (n=1), M4 (n=3), M5 (n=2), and M7 (n=1). Adult AML samples were from patients treated at the Ghent University Hospital, Ghent, Belgium (n=12, 71%) or VUmc, Amsterdam, the Netherlands (n=5, 29%). Belgian patients were treated according to local and international guidelines, whereas Dutch patients were included in the HOVON 102 (n=3) or HOVON 132 (n=2) study. Adults were risk stratified according to the European LeukemiaNet 2010 guidelines¹ and categorized according to the FAB classification into M1 (n=6), M2 (n=6), and M3 (n=2). *WT1* overexpression was interpreted according to in-house or published cut-offs. Core binding factor (CBF)-positive leukemias comprised AML with t(8;21)(q22;q22) (pedAML=3) and inv(16)(p13q22) (pedAML=1, adult AML=2). Other fusion transcripts detected were *DEK-NUP214* (pedAML=1) and *PML-RARA* (pedAML=1, adult AML=1). *One, †two, ‡three or ‖five missing data. BM: bone marrow; F: female; M: male; *NPM1*: nucleophosmin; PB: peripheral blood; WBC: white blood cell; WT1: Wilms' tumor 1.

ed synthetic TARP peptide TARP(P5L).⁴⁻¹³ Regarding RV transduced TARP-TCR CTL, mock CTL were used to correct for non-TARP mediated lysis, and CMV-TCR transduced CTL to evaluate aspecific killing.

Results

Discovery of T-cell receptor γ chain alternate reading frame protein transcript expression in acute myeloid leukemia

In order to identify LSC-specific antigens, we re-analyzed the gene set enrichment (GSE) 17054 micro-array dataset from Majeti *et al.*,³¹ which included gene expression profiles of CD34⁺CD38⁻ sorted fractions of four healthy adults (HSC) and nine adult AML patients (LSC). *TARP* ranked first amongst the top differentially expressed genes, with all four probes in the top 20 (range log2-FC 5.13-6.92), showing a significantly higher expression in LSC compared to HSC (*P*<0.01) (*Online Supplementary Figure S1*). TARP had previously been identified as a truncated TCR transcript expressed in androgen-sensitive

prostate and breast adenocarcinoma (*Online Supplementary Figure S2*).⁴¹,⁴² We further explored *TARP* expression in pedAML by micro-array profiling CD34⁺CD38⁺ (n=4, leukemic blast) and CD34⁺CD38⁻ (n=3, LSC) sorted cell populations from four pedAML patients (2 *FLT3*-ITD and 2 *FLT3* WT) (*Online Supplementary Table S1*). In addition, sorted CD34⁺CD38⁺ (n=3) and CD34⁺CD38- (n=2) cells from CB were profiled to examine the expression in their normal counterparts (*Online Supplementary Figure S3*). TARP appeared to be more highly expressed in leukemic blasts and LSC from *FLT3*-ITD patients compared to *FLT3* WT patients and CB (Figure 1A). This finding suggested that TARP might represent a LSC-associated target within HR pedAML patients harboring *FLT3*-ITD.

To validate these data in a larger patient group, we sorted CD34⁺CD38⁺ and CD34⁺CD38⁻ cell populations from nine additional pedAML (resulting in a total of 13 pedAML patients), 17 adult AML (Table 1) and 15 control samples (n=7 CB, n=6 NBM, n=2 mPBSC). qPCR analysis using TARP short primers (*Online Supplementary Table S3 and Online Supplementary Figure S2*) showed that *TARP* transcripts were consistently low in HSC and myeloblasts
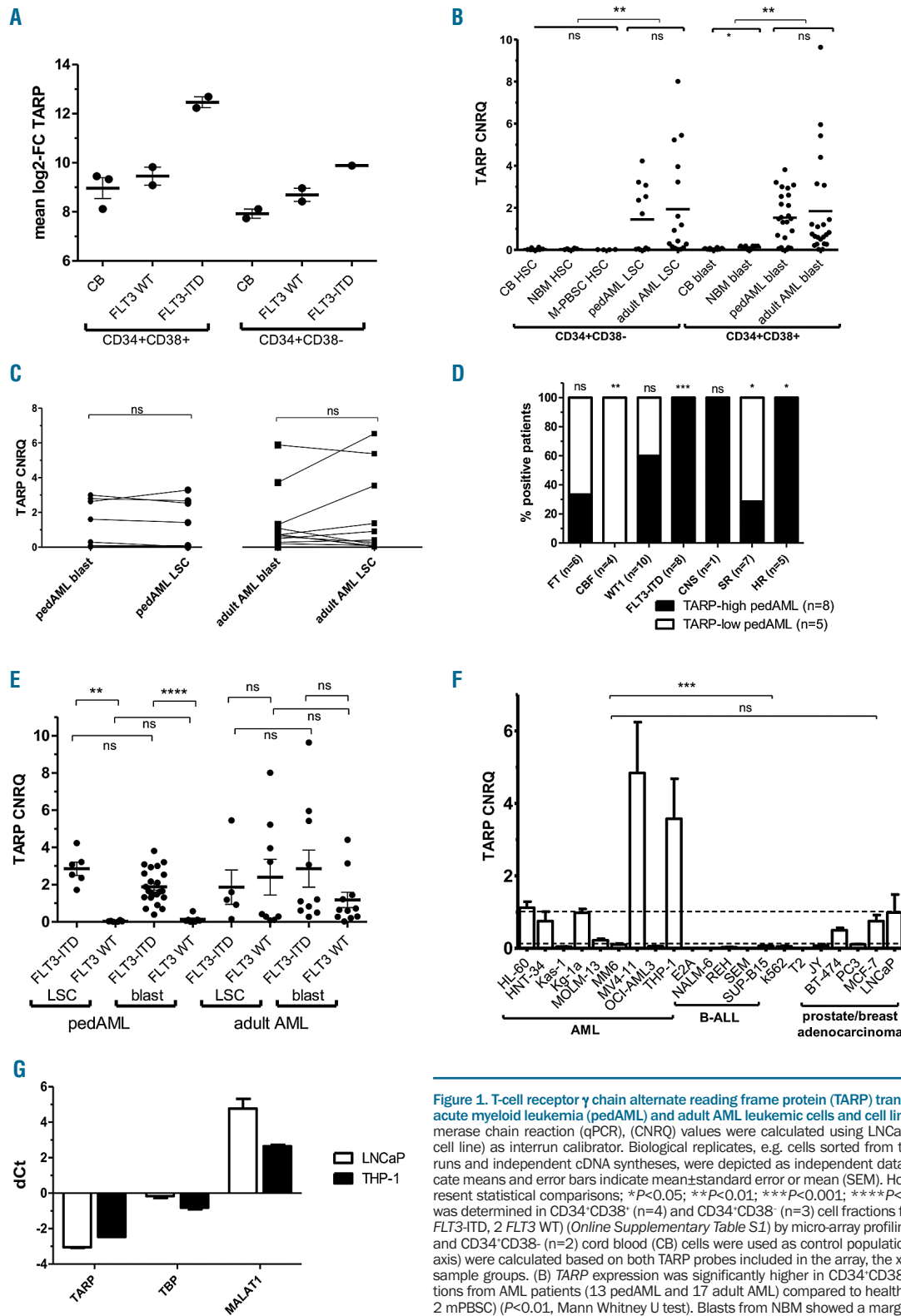
Figure 1. T-cell receptor γ chain alternate reading frame protein (TARP) transcript expression in pediatric acute myeloid leukemia (pedAML) and adult AML leukemic cells and cell lines. For TARP qualitative polymerase chain reaction (qPCR), (CNRQ) values were calculated using LNCaP (prostate adenocarcinoma cell line) as interrun calibrator. Biological replicates, e.g. cells sorted from the same patient in different runs and independent cDNA syntheses, were depicted as independent data points. Horizontal bars indicate means and error bars indicate mean±standard error or mean (SEM). Horizontal square brackets represent statistical comparisons; *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$. (A) TARP expression was determined in CD34+CD38+ (n=4) and CD34+CD38- (n=3) cell fractions from four pedAML patients (2 FLT3-ITD, 2 FLT3 WT) (Online Supplementary Table S1) by micro-array profiling. Sorted CD34+CD38+ (n=3) and CD34+CD38- (n=2) cord blood (CB) cells were used as control populations. Mean log2-FC values (y-axis) were calculated based on both TARP probes included in the array, the x-axis represents the different sample groups. (B) TARP expression was significantly higher in CD34+CD38- and CD34+CD38+ cell fractions from AML patients (13 pedAML and 17 adult AML) compared to healthy controls (7 CB, 6 NBM and 2 mPBSC) ($P<0.01$, Mann Whitney U test). Blasts from NBM showed a marginally higher expression compared to CB ($P=0.049$). (C) Comparison of TARP expression between leukemic stem cells (LSC) and blasts sorted from pediatric and adult AML patients on a per patient basis showed no significant differences ($P>0.05$, paired sample t-test). (D) Bars display the percentage of patients (%), harboring the characteristic shown in the x-axis (dichotomous variables, for details see Table 1), for TARP-high (black, n=8) and TARP-low (white, n=5) pedAML patients. The total number of patients positive for each characteristic is shown between parentheses. Patients without central nervous system (CNS) involvement all showed negative lumbar punctures. Data on CNS involvement and risk profile is lacking for one patient. The number of patients harboring FLT3-ITD ($P<0.001$) and HR profiles ($P<0.05$) were significantly higher in the TARP-high group, whereas TARP-low pedAML patients included significantly more CBF-leukemia ($P<0.01$) and SR profiles ($P<0.05$) (χ^2 test). (E) Differential TARP expression between LSC and blasts sorted from pediatric and adult AML patients with FLT3-ITD versus FLT3 WT. A significant higher TARP expression in LSC ($P<0.01$) and blasts ($P<0.0001$) was only detected for FLT3-ITD positive pedAML patients (Mann Whitney U test). (F) TARP expression in nine AML cell lines, five B-ALL cell lines, the CML cell line K562, the Epstein-Barr virus (EBV)-immortalized B-cell line JY and T2 cell line, next to two breast (BT-474, MCF-7) and two prostate (LNCaP, PC3) adenocarcinoma cell lines. Dashed lines indicate the expression observed in PC3 and LNCaP, serving as low and high reference, respectively, in agreement with previous literature.[41] (G) Delta (d) Ct values were calculated for TARP, MALAT1 and TBP between cytoplasmic and nuclear compartments of THP-1 and LNCaP, in order to examine the subcellular location of TARP. THP-1 showed a cytoplasmic residence for TARP, in agreement with LNCaP. FC: fold change; FT: fusion transcript; Kas-1: Kasumi-1; MM-6: MONO-MAC6; mPBSC: mobilized peripheral blood stem cells; NBM: normal bone marrow.
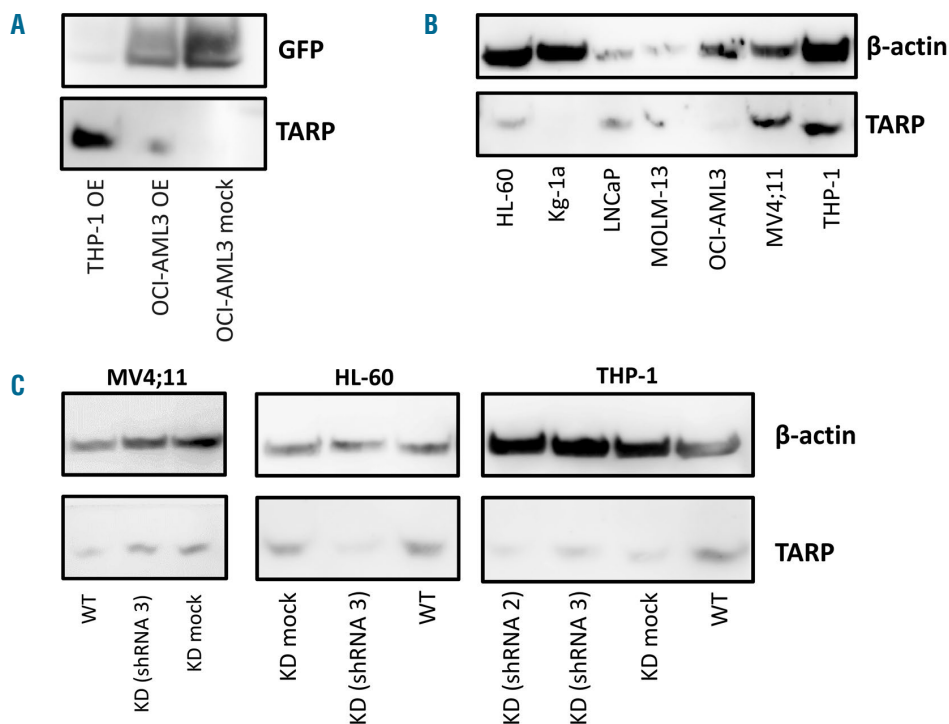
Figure 2. T-cell receptor γ chain alternate reading frame protein (TARP) expression in cell lines evaluated by western blotting. Whole-blot images with ladders used for size estimation are shown in *Online Supplementary Figure S7*. (A) TARP transgenic (OE) cell lines generated for OCI-AML3 and THP-1 showed a 27 kDa protein for GFP and a 15-25 kDa protein for TARP. In agreement with low TARP transcript levels, the OCI-AML3 mock cell line only showed a 27 kDa GFP protein. TARP expression in THP-1 OE was higher than OCI-AML3 OE, most likely resulting from both transgenic and cognate TARP protein expression, since THP-1 was categorized by qualitative polymerase chain reaction (qPCR) as a TARP-high acute myeloid leukemia (AML) cell line. (B) Immunoblotting of TARP and β-actin in AML cell lines (HL-60, Kg-1a, MOLM-13, OCI-AML3, MV4;11 and THP-1) next to LNCaP. Protein expression mostly matched transcript levels, except for Kg-1a, although confocal microscopy did allow TARP protein staining in Kg-1a. β-actin expression appeared to be lower for LNCaP and MOLM-13, although equal amounts of protein were loaded. (C) Immunoblotting of TARP and β-actin in selected shRNA-mediated knockdown (KD) AML cell lines for MV4;11, HL-60 and THP-1, next to their respective mock and wild-type (WT) cell line. For HL-60, a stable knockdown was introduced by shRNA 3 (19.4% compared to mock). KD: knockdown; OE: overexpression.

sorted from CB, NBM and mPBSC (Figure 1B), although blasts from NBM showed a marginally higher expression compared to CB (mean CNRQ 0.12 *vs.* 0.045, *P*=0.049). In sharp contrast, LSC and blasts from pediatric and adult AML showed significantly (*P*<0.01) higher expressions compared to their normal counterparts. Paired comparison between LSC and blasts on a per patient basis showed no significant differences (Figure 1C).

A cut-off for elevated TARP expression was determined based on the highest expression in control fractions plus two times the standard deviation. Classification of patients into TARP-high (8 pedAML, 13 adult AML) and TARP-low (5 pedAML, 4 adult AML) revealed that *FLT*3-ITD (*P*<0.001), CNS involvement and HR profile (*P*<0.05) were exclusively present in TARP-high pedAML patients (Figure 1D). TARP expression was shown to be significantly higher in sorted LSC (*P*<0.01) and blasts (*P*<0.0001) from *FLT*3-ITD compared to *FLT*3 WT pedAML (Figure 2E). In adult AML, high TARP expression was not restricted to *FLT*3-ITD. On the other hand, all pediatric (Figure 1D) and adult (*Online Supplementary Figure S4A*) core-binding factor (CBF) leukemia were classified as TARP-low patients (*P*<0.01). TARP-low pedAML patients were included in the standard risk (SR) groups (*P*<0.05). No significant differences in age, white blood cell (WBC) count, or blast percentages were observed

between TARP-high and -low pediatric or adult AML patients (*Online Supplementary Figure S4B and C*). We thus conclude that TARP is highly and specifically expressed in AML leukemic cells from both adults and children, showing a significant association with *FLT*3-ITD in pedAML.

Next, we evaluated TARP transcript levels in cell lines of various origin. Expression in breast and prostate adenocarcinoma (PC3, BT-474, LNCaP and MCF-7) was in agreement with previous findings[42] (Figure 1F). No expression was detected in five B-ALL cell lines, CML cell line K562, EBV-immortalized B-cell line JY and T2 cell line. Expression in AML cell lines, on the other hand, was significantly increased (*P*<0.001, one-way ANOVA). The highest expression was observed in HL-60, HNT-34, Kg-1a, MV4;11 and THP-1 (median CNRQ 1.12, range 0.75-4.84), whereas low transcript levels were observed in Kas-1, MOLM-13, MONO-MAC6 and OCI-AML3 (median CNRQ 0.080, range 0.049-0.22). Furthermore, fractionation revealed a mainly cytoplasmic localization of TARP mRNA in THP-1 (Figure 1G), as previously shown in LNCaP43.

To evaluate whether the TARP transcript detected in AML is identical to previous reports, we sequenced the TRGC region of different TARP amplicons obtained by qPCR for AML cell lines and pedAML leukemic cells. Using TARP long primers, we observed a single band for
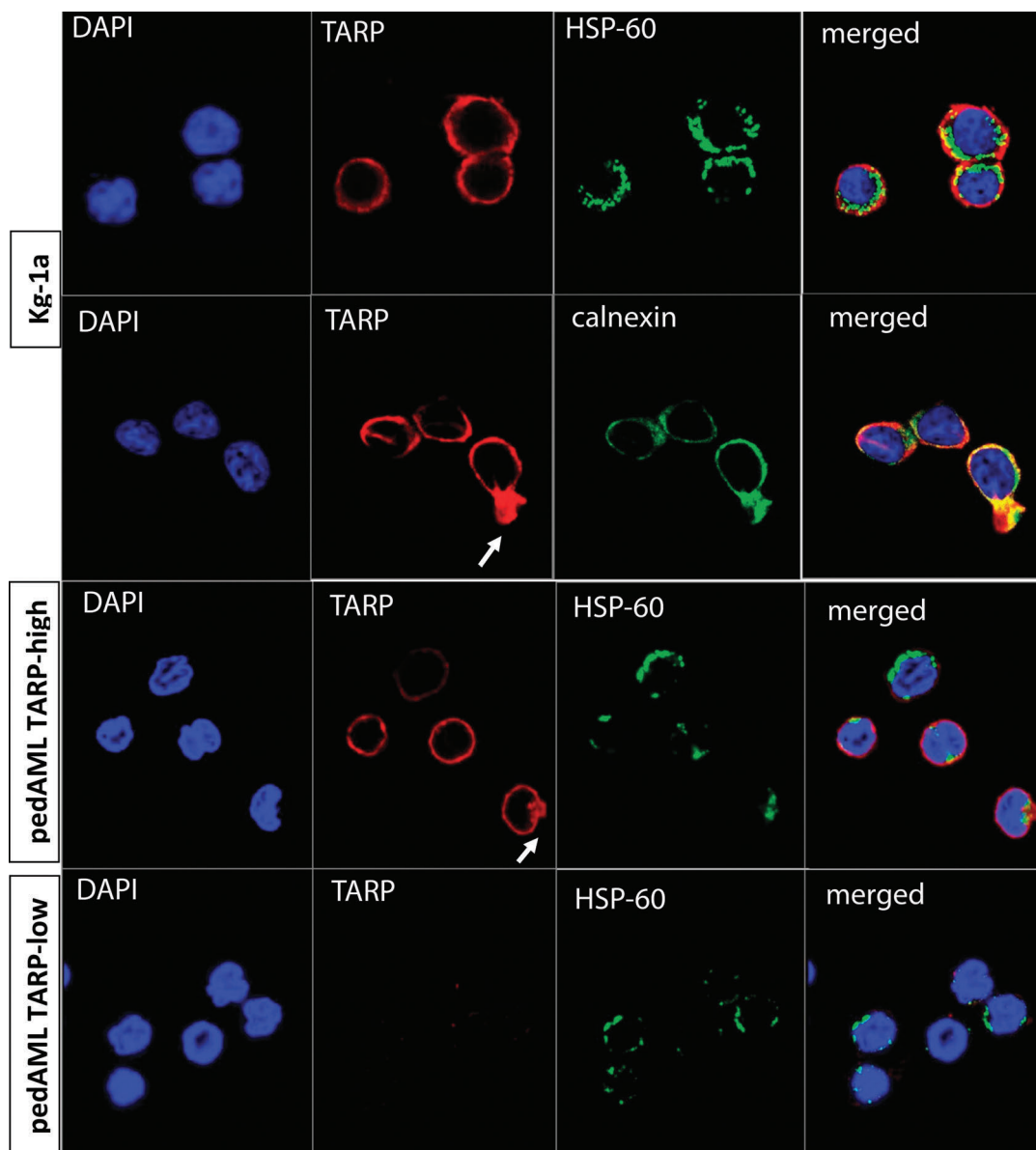
Figure 3. T-cell receptor γ chain alternate reading frame protein (TARP) protein detection in Kg-1a and patient leukemic cells by confocal microscopy. Merged patterns visualize TARP (red) and HSP-60 (top lane) or calnexin (bottom lane) (both in green) co-localization (yellow fusion signals) together with DAPI nuclear counterstaining (blue). Leukemic cells were sorted from two pediatric acute myeloid leukemia (pedAML) patients, classified as TARP-high and TARP-low by qualitative polymerase chain reaction. Calnexin staining was not performed on primary cells due to lack of material. Within Kg-1a and the sorted TARP-high leukemic cells, TARP expression was enriched at the cells' protrusions, indicated by arrows.

Kg-1a, which was similar to the LNCaP and TRGC1 reference sequence (*Online Supplementary Figure S5A*). Unexpectedly, three fragments were observed in the sorted blasts and LSC from TARP-high pedAML patients and the MV4;[11] cell line. Cloning and sequencing of each fragment (*Online Supplementary Figure S5B*) revealed that the largest fragments were artificial heteroduplexes,[44] whereas the smallest fragments were identical to the fragments from Kg-1a and LNCaP. Medium-sized fragments were consistently 48 bp longer, and showed the same size as the HSB-2 amplicon, a T-cell acute lymphoblastic leukemia (T-ALL) cell line with functional TRGC2 rearrangements.[45] As TRGC2 contains a duplicated second exon (48 bp) compared to TRGC145 (*Online Supplementary Figure S2*), we hypothesized that there might be an alternative TARP transcript in AML. Indeed, most AML cell lines, but none of the prostate and breast adenocarcinoma cell lines, showed TRGC1 as well as TRGC2 amplicons (*Online Supplementary Figure S5C and E*). Single bands for exon 3 and exon 1 amplicons in all cell lines provided evidence that the occurrence of the second transcript is related to the TRGC2 duplicated second exon. Altogether, TARP was highly expressed in approximately half of the AML cell lines evaluated, and both TRGC1- and TRGC2-related transcripts co-existed in AML.
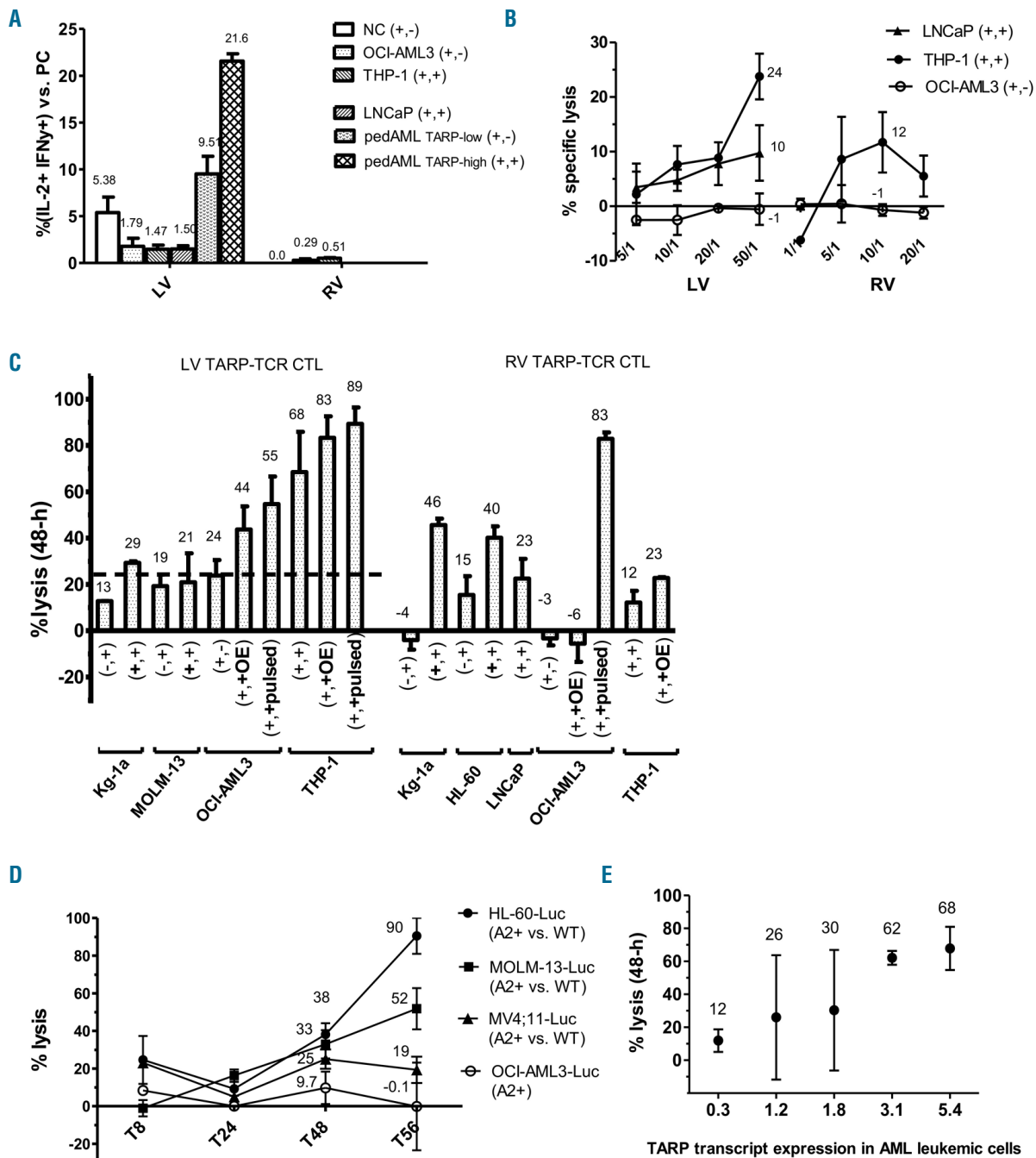
Figure 4. Functional evaluation of T-cell receptor (TCR)-transgenic cytotoxic T cells (CTL) towards cognate and modified cell lines and patient leukemic cells. (A) Cytokine response (IFN-γ/IL-2 expression within the CD3+/CD8+ compartment) by co-incubation (1 hour, h) with OCI-AML3 and THP-1 was evaluated by both lentiviral (LV) and retroviral (RV) TCR-T-cell receptor γ chain alternate reading frame protein (TARP) CTL. LNCaP and patient leukemic cells (single experiment) were only evaluated by LV transduced TARP-TCR CTL. For each target, positive (+) or negative (-) HLA-A*0201 and TARP expression, in this respective order, is indicated between brackets. HLA-A*0201 and TARP co-expressing cell lines (LNCaP and THP-1) were unable to trigger higher cytokine release than OCI-AML3 with low TARP expression. Leukemic cells from a TARP-high pediatric acute myeloid leukemia (pedAML) patient triggered a 2-fold higher cytokine release compared to a TARP-low pedAML patient. (B) Lytic response of LV and RV TARP-TCR CTL *versus* HLA-A*0201-positive TARP-high (black symbols) and TARP-low (white symbols) targets, measured by a chromium[51] release assay after 4 h. Highest lysis of TARP-high cell lines was observed at E/T ratio 50/1 for LV and 10/1 for RV TARP-TCR CTL (percentages indicated between brackets), whereas OCI-AML3 (HLA-A*0201+, TARP-) remained unaffected. (C) Lytic response of LV and RV TARP-TCR CTL *versus* towards wild-type (WT), transgenic and pulsed AML cell lines, measured by a 48-h FCM-based cytotoxicity assay. The dashed line indicates the highest level of non-TARP mediated background killing observed for LV TARP-TCR CTL, as no mock CTL could be constructed. Positive (+) or negative (-) expression for HLA-A*0201 and TARP is shown, in this respective order, between brackets. Bold symbols indicate the expression differing from wild-type, either by retroviral transduction or pulsing. HLA-A*0201 transgenic AML cell lines were more efficiently lysed compared to their HLA-A*0201-negative counterparts (Kg-1a, MOLM-13, HL-60). Higher lysis was observed for transgenic TARP OE or peptide-pulsed cell lines compared to the WT cell line (OCI-AML3, THP-1), except for killing of TARP OE OCI-AML3 cell line by RV TARP-TCR CTL. (D) Lysis by LV TARP-TCR CTL, measured at different time points (8h, 24h, 48h and 56h, as indicated on x-axis), based on the luminescence release by transgenic HLA-A*0201-expressing TARP-high AML cell lines with respect to the HLA-A*0201 WT cell line (HL-60-Luc, MOLM-13-Luc and MV4;11-Luc: black symbols). In addition, lysis of the TARP-low, cognate HLA-A*0201-positive OCI-AML3 cell line was evaluated (white symbols). Mean lysis (%) observed after 48 h is indicated next to whiskers, representing the mean±standard error of mean. (E) 48-h FCM-based cytotoxicity assay evaluating lysis of primary leukemic cells (adult AML=5, all FLT3-ITD mutated) by LV TARP-TCR transduced CTL (biological duplicates). TARP transcript expression (CNRQ) is shown in the x-axis for each target. IFN-γ: interferon gamma; IL-2: interleukin-2; INF: influenza.

T-cell receptor γ chain alternate reading frame protein is expressed in acute myeloid leukemia cell lines and patient leukemic cells

We generated TARP transgenic cell lines in order to optimize western blot experiments and evaluate TARP protein expression in AML. THP-1 and OCI-AML3 OE cell lines showed a significant higher *TARP* transcript expression ($P<0.01$) compared to mock controls (*Online Supplementary Figure S6A*). Western blotting confirmed presence of TARP and GFP proteins in both OE cell lines, with a size of around 20 kDa and 27 kDa, respectively (Figure 2A and *Online Supplementary Figure S7*). Concordantly, the OCI-AML3 mock cell line, negative for TARP, only showed a 27 kDa GFP protein. WT AML cell lines HL-60, MV4;11, THP-1 and MOLM-13, as well as LNCaP, also showed a 20 kDa TARP protein, with expression corresponding to the transcript levels (Figure 2B and *Online Supplementary Figure S7*). TARP knockdown (KD) cell lines were generated for HL-60, Kg-1a, MV4;11 and THP-1 using TARP-targeting shRNA, next to mock controls. Transcript levels were efficiently down-regulated (*Online Supplementary Figure S8*), and KD cell lines for HL-60, MV4;11 and THP-1 showing the highest transcript downregulation were selected for western blotting (Figure 2C and *Online Supplementary Figure S7*). Protein levels were efficiently down-regulated in HL-60 transduced with shRNA 3 (19.4% compared to mock). This downregulation was less clear in MV4;11 and THP-1: 116% (shRNA 3) and 108% (shRNA 3) *versus* 63% (shRNA 2), respectively.

To confirm western blot data and determine the subcellular location of TARP, confocal microscopy was performed using TARP antibodies combined with mitochondrial (HSP-60) and endoplasmic reticulum (ER, calnexin) staining. The over-expressing OCI-AML3 and THP-1 cell lines (*Online Supplementary Figure S6B and C*) and TARP-high WT AML cell lines showed a perinuclear membranous-type TARP staining pattern (Kg-1a (Figure 3), HL-60, MV4;11 and THP-1 (*Online Supplementary Figure S9*). This finding was in contrast to the barrel-shaped TARP pattern with mitochondrial co-localization reported in LNCaP.[43] Co-localization with calnexin, presenting as a speckled pattern throughout the ER, was more abundant in some cell lines, e.g. Kg-1a, showing TARP enrichment at the cells' protrusions. TARP-low cell lines concordantly showed weak or negative TARP protein staining (*Online Supplementary Figure S8*). Importantly, the leukemic cells sorted from a TARP-high and TARP-low pedAML patient also illustrated differential TARP protein expression in agreement with the transcript levels, again showing limited mitochondrial overlap (Figure 3).

T-cell receptor γ chain alternate reading frame protein transgenic cytotoxic T cells display specific anti-leukemic activity

To explore if TARP might represent an immunotherapeutic target in AML, we evaluated the cytokine and cytotoxicity response of TARP-TCR transgenic CTL, encoding a previously developed TCRA8-T2A-TCRB12 sequence targeting the HLA-A2 enhanced affinity TARP(P5L)$_{4-13}$ epitope.[46,47] As concomitant HLA-A*0201 and TARP expression is required to trigger TCR-mediated killing, HLA-A*0201 transgenic cell lines were generated for 3 WT cell lines (HL-60, Kg-1a and MOLM-13) and 3 Luc-positive cell lines (HL-60-Luc, MOLM-13-Luc, MV4;11-Luc).

First, target specificity of the TARP-TCR was examined in a non-competitive environment using T2 cells (endogenous HLA-A*0201+) pulsed with exogenous peptides (*Online Supplementary Table S3*). As expected, we found stronger cytokine responses (*Online Supplementary Figure S10A*) and higher killing rates (*Online Supplementary Figure S10B and C*) towards the TARP(P5L)$_{4-13}$ than to the cognate TARP$_{4-13}$ peptide for both RV and LV transduced CTL, with LV TARP-TCR CTL generally reacting stronger. T2 cells pulsed with non-TARP-related peptides (INF, CMV) were not affected, although CMV-pulsed T2 cells were efficiently recognized by CMV-TCR CTL, indicating a high specificity of the TARP-TCR.

Second, we explored the immunogenicity of cell lines with endogenous HLA-A*0201 presentation. Exposure to LNCaP and THP-1 appeared to be insufficient to trigger cytokine release for both LV and RV transduced TARP-TCR CTL (Figure 4A). Using a chromium[51] release assay, we observed a lytic response by LV transduced TARP-TCR CTL starting from effector to target ratio (E/T) 10/1, with a maximal average response at 50/1 (LNCaP 10%, THP-1 24%), whereas RV transduced TARP-TCR CTL performed best at 10/1 (THP-1 12%) (Figure 4B). The TARP-low AML cell line OCI-AML3 remained unaffected under all conditions. Altogether, as the TARP-TCR targets the enhanced HLA-A2 TARP(P5L)$_{4-13}$ binding peptide, we observed weaker responses against endogenous TARP-expressing cell lines compared to pulsed T2 cells.

Third, lysis of TARP-high HLA-A*0201-negative cell lines was evaluated *versus* their HLA-A*0201 transgenic counterparts in a 48-h FCM-cytotoxicity assay. In addition, killing of TARP transgenic or TARP-pulsed HLA-A*0201-positive cell lines was compared to the respective TARP-low WT cell line (Figure 4C). A non-TARP mediated lysis by LV TARP-TCR CTL of maximal 20% was observed (indicated by dashed line). Stable transduction of HLA-A*0201 increased killing for Kg-1a compared to the WT cell line (29% *vs.* 13%), whereas killing of MOLM-13, with lower TARP expression levels, remained unaffected when HLA-A*0201 was introduced. Transgenic TARP OE and TARP(P5L)$_{4-13}$ pulsed OCI-AML3 cells were prone to a higher lysis than the WT cell line (44% and 55%, respectively, *vs.* 24%). Killing of TARP OE/pulsed THP-1 cells was only marginally up-regulated, most likely due to an already high endogenous expression. These data were confirmed using RV TARP-TCR CTL, and corrected for non-TARP mediated lysis using mock CTL. HLA-A*0201 expression again increased killing of Kg-1a (46% *vs.* -4%) and HL-60 (40% *vs.* 15%) compared to the WT cell line. Up-regulated killing of transgenic TARP OE THP-1 cells was again limited. For OCI-AML3, lysis was up-regulated after pulsing, but remained low for the TARP OE transgenic cell line. Killing by LV TARP-TCR CTL was additionally evaluated in a bioluminescence imaging (BLI)-based assay using Luc-positive AML cell lines with high TARP expression (HL-60 and MV4;11) and low TARP expression (MOLM-13 and OCI-AML3) (Figure 4D). A higher lysis was observed for HL-60-Luc and MV4;11-Luc when expressing HLA-A*0201 at 48 h and 56 h, indicating that also in long-term cytotoxicity experiments HLA-A*0201 restricted TARP-specific killing could be detected.

Finally, we explored the feasibility of therapeutic targeting of primary leukemic cells by LV TARP-TCR CTL. Co-incubation with blasts sorted from a TARP-high pedAML patient resulted into a 2-fold higher IFN-γ and IL-2 production compared to a TARP-low pedAML patient (22%

vs. 10%) (Figure 4A). Moreover, TARP-TCR CTL were also capable of killing leukemic cells from *de novo* adult AML patients (n=5) (Figure 4E). Lysis ranged between 12% and 68%, and borderline correlated to *TARP* transcript levels (Spearman's coefficient 0.82, *P*=0.089).

Discussion

We demonstrated increased *TARP* expression in AML LSC (CD34+CD38−) and blasts (CD34+CD38+) from primary patients compared to their normal counterparts as well as AML cell lines. We also showed that TARP protein is expressed in primary AML leukemic cells and are adequately presented on HLA molecules, which makes the cells targetable for immunotherapy.

TARP expression has only been investigated in prostate tissue and androgen-sensitive prostate adenocarcinoma and breast adenocarcinoma,[42,43,48] next to a single report on salivary adenoid cystic carcinoma.[49] We found that *TARP* expression was significantly (*P*<0.001) higher in *FLT3*-ITD compared to *FLT3* WT pedAML patients at diagnosis, whereas no significant difference was observed in adult AML. Importantly, enormous differences in the genomic landscape in adult compared to pedAML were shown,[50,51] potentially explaining some of the differential associations observed in our cohorts. The association between *TARP* expression and a poor prognosis is in agreement with a recent report, investigating the association between transcript expression and clinical outcome in pedAML, ranking *TARP* within the top genes significantly associated with a detrimental outcome.[52] To shed light on the link between *FLT3*-ITD and *TARP*, mRNA sequencing of the transgenic OE and KD cell lines compared to their WT cell line is ongoing. As it was recently shown that the *FLT3*-ITD regions encode immunogenic, HLA-presented neo-epitopes,[53] the benefit of CTL therapy targeting both leukemogenic molecules in pedAML could be of great interest. On the other hand, CBF leukemias, representing a favorable cytogenetic subgroup,[2,8] were only present (*P*<0.01) in the TARP-low group for both pediatric and adult patients. AML cell lines derived from pediatric cases (MV4;11, THP-1) and LSC-enriched cell lines (Kg-1a, HNT-34), showed the highest TARP levels, confirming a relation between TARP, the LSC compartment and pedAML, although also HL-60 (adult, CD34−) showed high expression. Whether TARP remains differentially expressed within LSC outside the predominant CD34+CD38− compartment, as it does within CD34− AML,[15,54] needs to be explored further. In addition, we showed that transcripts differ from those in solid tumors and are derived from both the TRGC1 and TRGC2 coding regions. Sequencing analysis indicated the presence of a second, AML-exclusive, TARP transcript encoding TRGC2 instead of TRGC1.

TARP protein expression was in agreement with transcript levels, showing a 15-25 kDa fragment in AML cell lines. In breast and prostate adenocarcinoma, TARP had previously been defined as a 7 kDa protein,[42,48] although also a 9 kDa fragment was reported in MCF-7.[42,48] Fritzsche *et al.* detected protein sizes in prostate carcinoma of 20-25 kDa,[55] comparable to our findings, whereas Yue *et al.* reported a 15 kDa protein.[49] Besides its size, the subcellular localization of TARP in AML needs to be refined. qPCR analysis revealed cytoplasmic localization,

and confocal microscopy showed sporadic ER overlap, in contrast to previously reported mitochondrial co-localization.[43] We observed an enrichment of TARP at the cells' protrusions in Kg-1a and sorted leukemic cells. Protrusions are kinetic cytoskeletal abnormalities formed during chemokine-induced cell migration, e.g. homing of CD34+ HSC towards the bone marrow niche.[56] The presence of molecular abnormalities in CD34+ progenitor cells was shown to increase protrusion formation.[57] Indeed, LSC were reported to compete with HSC for endosteal niche engraftment, where they are protected from chemotherapy-induced apoptosis.[12,58] Whether TARP interferes in homing and chemoprotection of leukemic AML cells in the BM microenvironment needs to be elucidated. Although protein expression was readily up-regulated in TARP transgenic cell lines, shRNA-mediated knockdown appeared to be less efficient. Possible explanations are the presence of escape mechanisms and alternative translation pathways during silencing or a very high stability of the TARP protein, persisting in the cell for a long period of time.

To explore TARP as an immunotherapeutic target in AML, we evaluated the cytokine release and cytotoxic killing capacities of TARP-TCR transgenic CTL *in vitro*. TARP and HLA-A*0201 co-expressing cell lines were efficiently lysed, and although evaluated on a limited number of patients (n=5), TARP-TCR CTL were able to kill primary leukemic cells with a borderline correlation to the *TARP* transcript expression. Interestingly, weaker responses were observed for the cognate TARP$_{4-13}$ peptide, since the TCR is directed against the HLA-A*0201 enhanced affinity TARP(P5L)$_{4-13}$ peptide. Moreover, pulsed T2 cells appeared to be more susceptible than AML cells. This finding is in agreement with previous data,[47,59] and several reasons may account for this phenomenon. First, peptide processing, transport and/or MHC-I presentation may be disturbed in leukemic cells.[60] Second, high and stable HLA-A*0201 expression is vital for triggering lytic responses, and transgenic expression might diminish during culture. Therefore, we cannot exclude the possibility that HLA-A*0201-mediated TARP presentation within the transgenic OCI-AML3 cell line had diminished during long-term culture. Third, competition between transgenic and endogenous MHC-I molecules might block HLA-A*0201-guided peptide presentation. Indeed, the TARP-TCR was shown to suffer from low MHC-I avidity compared to foreign epitope-directed TCR.[61] Cloning the TARP$_{4-13}$-TCR sequence into a retroviral construct enabled higher transduction efficiencies and the generation of mock CTL to correct non-TARP mediated lysis, which are lacking in previous reports.[37,49] As promoters driving TCR expression differed between constructs, and functional activity is known to correlate with TCR cell-surface expression,[62] it was no surprise that different killing rates between LV and RV transduced CTL were osberved. In addition, intrinsic reactivity, HLA status and endogenous TCR repertoire of each donor as such might have an impact.[62] In addition, comparing reactivity by effectors from an allogeneic *versus* autologous setting will be implemented in future experiments.

In conclusion, we showed that *TARP* is highly expressed in AML leukemic cells, including the CD34+CD38− LSC compartment, while absent in normal counterparts. Moreover, *TARP* expression was associated with *FLT3*-ITD in pedAML. We provide *in vitro* evidence that

TARP-directed CTL effectively kill TARP and HLA-A*0201 co-expressing cell lines and primary leukemic cells, and thus hold great promise for immunotherapeutic T-cell therapy.

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