

T cells engineered with a T cell receptor against the prostate antigen TARP specifically kill HLA-A2⁺ prostate and breast cancer cells

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To produce genetically engineered T cells directed against prostate and breast cancer cells, we have cloned the T-cell receptor recognizing the HLA-A2–restricted T-cell receptor γ -chain alternate reading-frame protein (TARP)_{4–13} epitope. TARP is a protein exclusively expressed in normal prostate epithelium and in adenocarcinomas of the prostate and breast. Peripheral blood T cells transduced with a lentiviral vector encoding the TARP-TCR proliferated well when exposed to peptide-specific stimuli. These cells exerted peptide-specific IFN- γ production and cytotoxic activity. Importantly, HLA-A2⁺ prostate and breast cancer cells expressing TARP were also killed, demonstrating that the TARP_{4–13} epitope is a physiologically relevant target for T-cell therapy of prostate and breast cancer. In conclusion, we present the cloning of a T cell receptor (TCR) directed against a physiologically relevant HLA-A2 epitope of TARP. To our knowledge this report on engineering of T cells with a TCR directed against an antigen specifically expressed by prostate cells is unique.

genetic engineering | T-cell receptor transfer

T-cell-based immunotherapy is a promising approach to treat disseminated cancer; however, it has been limited by the difficulty to isolate and expand T cells specific for tumor-associated antigens. Using ex vivo lentiviral gene transfer, patient T cells can be genetically engineered to express a novel T-cell receptor (TCR) or chimeric antigen receptor (CAR). Thereby, they acquire specificity for tumor-associated antigens and hence exert tumor cell cytotoxicity. Indeed, T cells genetically engineered with TCRs or CARs have recently been successfully used in cancer treatment (1–3). In terms of prostate cancer, CARs against the prostate-specific membrane antigen (PSMA) (4, 5) and the prostate stem cell antigen (PSCA) (6) have been evaluated preclinically. Genetically engineered T cells with a TCR directed against a prostate differentiation antigen have not yet been developed.

The TCR γ chain alternate reading-frame protein (TARP) is a protein exclusively expressed in normal prostate epithelium, as well as in adenocarcinomas of the prostate and breast (7, 8). Three HLA-A2–restricted TARP epitopes with relevance for cancer vaccine, and cytolytic T-cell development have been described: TARP_{4–13} (9), TARP_{27–35} (9, 10), and TARP_{29–37} (10). T-helper cell epitopes derived from TARP have also been reported (11) and an antibody fragment against the HLA-A2/TARP_{29–37} complex has been developed for targeting TARP-expressing cancer cells (12). Antibody responses against TARP have been detected in prostate cancer patient treated with GM-CSF–secreting cellular immunotherapy (13) and early-stage HLA-A2⁺ prostate cancer patients have circulating CD8⁺ T cells against the TARP_{4–13} and TARP_{27–35} epitopes (14). Taken together, these data indicate that TARP is a relevant immunological target in prostate cancer. We have successfully used tetramers with HLA-A2–restricted TARP peptides to isolate and expand TARP-specific CD8⁺ T cells ex vivo in the past (9). We have now used limited dilution of TARP-specific T cells and cloned a TCR specific for the HLA-A2–restricted TARP_{4–13} epitope (FPPSPLFFFL). The coding sequence for the full-length TCR α and β chains with human variable (V), diverse

(D), and joining (J) segments and mouse constant domains (C) was synthesized and cloned into a lentiviral vector. T cells engineered with the unique TARP_{4–13}–specific TCR were found to be specific and efficient in killing TARP-expressing HLA-A2⁺ prostate and breast cancer cells.

Results

We developed single-cell clones of sorted and long-term stored TARP_{4–13}–specific T cells (9) through limited dilution. RNA was isolated from TARP(P5L)_{4–13}/HLA-A2-dextramer–positive clones that also secreted high levels of IFN- γ against T2 cells pulsed with the TARP(P5L)_{4–13} peptide. Ten individual clones were sequenced and verified to have identical TCR α chains and identical TCR β chains. The sequence encoding the developed TCR was synthesized and cloned in a lentiviral vector under transcriptional control of the spleen focus forming virus (SFFV) promoter (Fig. 1A). To ensure equimolar expression of TCR α and TCR β , the sequence encoding the self-cleaving peptide T2A was introduced in between the two chains. To reduce mispairing with endogenous TCR α and β chains, the constant domains of the introduced TCR α and β chains were replaced with mouse counterparts.

Human peripheral blood CD8⁺ lymphocytes were transduced with the TARP-TCR–encoding lentivirus and 1 wk later were stained with antibodies against CD3 and CD8 and with the TARP (P5L)_{4–13}/HLA-A2 dextramer. The dextramer positivity (Fig. 1B, after transduction) verifies the specificity of the cloned TCR, and it shows that the introduced TCR α and β chains pairs efficiently with each other and are expressed on the surface of transduced T cells. The transduced T cells were then rapidly expanded 500-fold and reexamined with the TARP(P5L)_{4–13}/HLA-A2 dextramer. The percentage of TARP-TCR–positive cells and TARP-TCR expression level remained unchanged (Fig. 1B, after REP), indicating stable expression of the transferred TCR. TARP-TCR–engineered T cells produced IFN- γ when cocultured with T2 cells pulsed with the TARP(P5L)_{4–13} peptide (Fig. 2A, T2 TARP) but not when cocultured with T2 cells pulsed with the irrelevant HIV-1p_{1476–484} peptide (Fig. 2A, T2 HIV). A peptide-pulsing time of the target cells of 2 h was sufficient for high levels of IFN- γ production from the TARP-TCR–engineered T cells (Fig. S1). Furthermore, the TARP-TCR–engineered T cells specifically produced IFN- γ when cocultured with HLA-A2⁺ tumor cells transfected with a plasmid encoding the wild-type TARP protein (Fig. 2B, mel526 TARP) but not when cocultured with the same tumor cells transfected with an irrelevant PSCA-expressing

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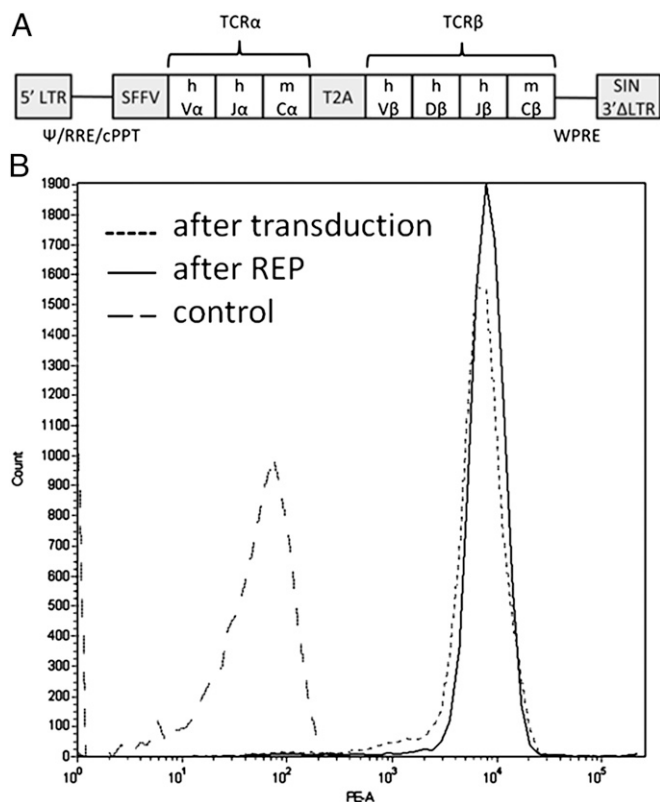


Fig. 1. Illustration of the TARP-TCR expression cassette and TARP-TCR expression in engineered T cells. (A) The encoded TCR, which specifically recognizes the HLA-A2/TARP₄₋₁₃ complex, has TCR α and TCR β chains with human (h) variable (V) and mouse (m) constant (C) domains. The TCR α and TCR β sequences are separated by a sequence encoding a self-cleaving T2A peptide and the SFFV promoter controls expression of the TCR transgene in a lentiviral vector. (B) Peripheral blood lymphocytes were transduced with the TARP-TCR-encoding lentiviral vector and CD3⁺, CD8⁺ T cells expressing the TCR were detected by TARP(P5L)₄₋₁₃/HLA-A2-dextramer after transduction and after REP expanding the T cells. Control cells did not show unspecific binding to the dextramer. The experiment was repeated at least three times and one representative experiment is shown.

plasmid (Fig. 2B, mel526 PSCA). The specific secretion of IFN- γ from TARP-TCR-engineered T cells when mixed with HLA-A2⁺ tumor cells transfected with a wild-type TARP-expressing plasmid is evidence of intracellular processing of the TARP protein yielding TARP₄₋₁₃ peptide presentation by HLA-A2. Importantly, this evidence shows that the transferred TCR, which binds the TARP₄₋₁₃/HLA-A2 complex, recognizes a physiologically relevant peptide epitope.

Next, we evaluated the proliferative capacity of the TARP-TCR-engineered T cells. These cells were labeled with 5- (and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured for 5 d with HLA-A2⁺ target cells presenting either the relevant TARP(P5L)₄₋₁₃ peptide or the irrelevant HIV-1pol₄₇₆₋₄₈₄ peptide. We observed efficient and specific proliferation of the TARP-TCR-engineered T cells after stimulation with TARP-presenting target cells (Fig. 3A). The data show that TARP-TCR-engineered T cells can proliferate efficiently upon contact with their cognate antigen.

Surface localization of the degranulation marker CD107a, also known as LAMP-1, which is normally found inside granules of T cells, is a sign of cytolytic activity as the T cells release perforin and granzymes from the granules to kill target cells. Specific and significant up-regulation of CD107a was observed on the TARP-TCR-engineered T cells after exposure to target cells pulsed with

the TARP(P5L)₄₋₁₃ peptide (Fig. 3B and D). A clear—however, not significant—up-regulation was also observed after exposure to target cells overexpressing the TARP protein (Fig. 3C and D). A chromium release assay confirmed that the TARP-TCR-engineered T cells specifically kill TARP(P5L)₄₋₁₃ peptide-pulsed T2 target cells (Fig. 4A). Furthermore, using the same assay we were able to demonstrate specific killing of HLA-A2⁺ tumor cells transfected with a plasmid encoding the full-length wild-type TARP (Fig. 4B). HLA-A2-restricted cytotoxicity was further verified by a flow cytometry-based assay where TARP plasmid-transfected HLA-A2⁺ mel526 cells were killed but TARP plasmid-transfected HLA-A2⁻ HEK-293T cells were not killed (Fig. 4C).

There is today no appropriate prostate cancer cell line expressing TARP along with high levels of HLA-A2 (15). The prostate cancer cell line LNCaP expresses TARP (15), (Fig. S24) and is HLA-A2⁺ (15). However, LNCaP has an intracellular defect in the assembly of the MHC class I molecule (16) and HLA-A2 expression on the cell surface is virtually zero and is not increasing after IFN- γ treatment (15, 16). Herein, we overcame the low HLA-A2 surface expression by engineering LNCaP to express HLA-A2 from a lentiviral vector (Fig. 2SB). The breast cancer cell line MCF7 is both positive for TARP (15) (Fig. S24) and HLA-A2⁺ (15). Upon IFN- γ treatment, MCF7 up-regulates its surface expression of HLA-A2 (15), (Fig. S2C). To evaluate whether the TARP-TCR-engineered T cells were able to kill prostate and breast cancer cells that naturally express TARP, we tested their ability to kill LNCaP and MCF7. Killing of target cells by TARP-TCR-engineered T cells was observed at ratios 10:1 and 50:1 (Fig. 4D, TCR T), whereas non-engineered T cells were not able to kill target cells even at 50:1 ratio (Fig. 4D, control T). The data presented in Figs. 3C and 4B–D further stress that TARP is processed in such a way that the TARP₄₋₁₃ peptide is presented in the context of HLA-A2 on the surface of target cells, showing that TARP₄₋₁₃ is a physiologically relevant target for T-cell therapy of prostate and breast cancer.

Discussion

Recent clinical trials for prostate cancer have demonstrated that immunotherapy can lead to improvements in overall survival. These

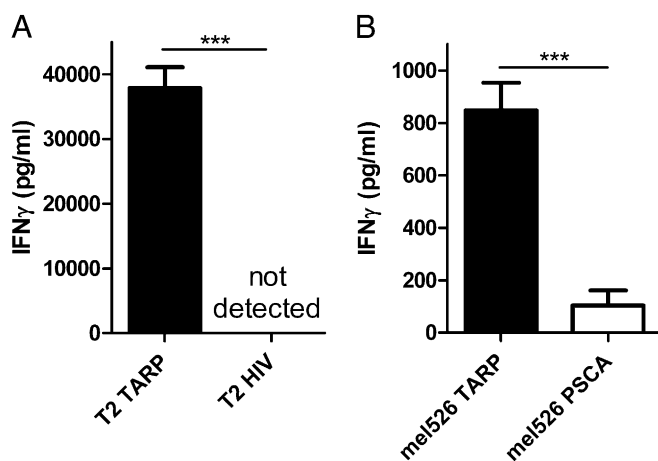


Fig. 2. TARP-TCR-modified T cells secrete IFN- γ in response to peptide-specific stimulation. (A) HLA-A2⁺ T2 target cells were pulsed for 2 h with the TARP(P5L)₄₋₁₃ peptide (T2 TARP) or the irrelevant HIV-1pol₄₇₆₋₄₈₄ peptide (T2 HIV) and cocultured overnight with TARP-TCR-engineered T cells. (B) HLA-A2⁺ tumor cells (mel526) were transfected with a plasmid encoding either the full-length wild-type TARP protein (mel526 TARP) or the irrelevant PSCA protein (mel526 PSCA) and cocultured overnight with TARP-TCR-engineered T cells. An ELISA was used to measure IFN- γ secretion from TARP-TCR-engineered T cells. Asterisks denote significant difference (*** $P < 0.001$, Student t test). Bars represent mean \pm SD from three experiments.

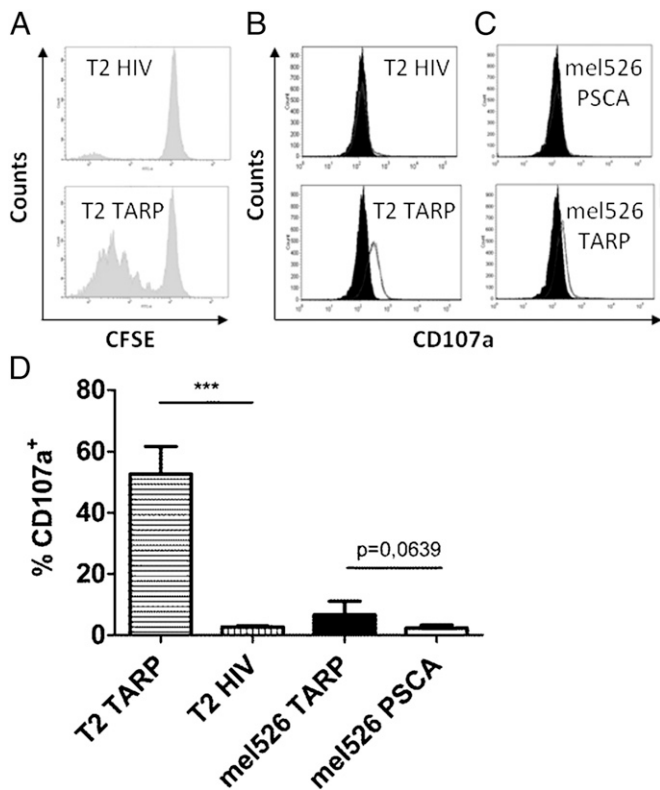


Fig. 3. TARP-TCR-modified T cells proliferate and get activated in response to specific stimulation. (A) TARP-TCR-engineered T cells were labeled with CFSE and mixed with T2 target cells pulsed with either the HLA-A2-restricted TARP (P5L)₄₋₁₃ peptide (T2 TARP) or the irrelevant HLA-A2-restricted HIV-1_{pol}₄₇₆₋₄₈₄ peptide (T2 HIV). The CFSE-labeled T cells were analyzed for cell proliferation by flow cytometry 5 d later. One representative experiment of three is shown. (B) TARP-TCR-engineered T cells were mixed with T2 target cells pulsed with either the TARP(P5L)₄₋₁₃ peptide (T2 TARP) or the irrelevant HIV-1_{pol}₄₇₆₋₄₈₄ peptide (T2 HIV). T cells were analyzed for CD107a expression after 12 h. One representative experiment of two is shown. (C) TARP-TCR-engineered T cells were mixed with mel526 target cells transfected with a plasmid encoding either the full-length wild-type TARP (mel526 TARP) protein or the irrelevant PSCA protein (mel526 PSCA). T cells were analyzed for CD107a expression after 12 h. One representative experiment of two is shown. (D) Percentage CD107a⁺ of all CD3⁺ T cells after culture with the indicated target cells. Asterisks denote significant difference (***) $P < 0.001$, Student *t* test). Bars depict mean \pm 5D of two experiments run in triplicates.

studies include randomized controlled trials with Provenge and PROSTVAC-VF, both of which rely on stimulating the immune system to target prostate proteins (17). Furthermore, the success story of genetically engineered T cells inducing complete remission in patients with otherwise treatment refractory B-cell leukemia (1, 3) indicates that T-cell therapy may lead to efficient new treatment options for patients with incurable cancer. Genetically engineered T cells with CARs against PSMA have recently entered clinical trials (www.clinicaltrials.gov). However, so far TCR-engineered T cells have not yet been developed for prostate cancer.

Herein, we present a unique report on the cloning of a TCR with specificity for a prostate differentiation antigen. The targeted antigen is TARP, a protein exclusively expressed in normal prostate epithelium, as well as in adenocarcinomas of the prostate and breast. TARP may be a particularly good target for T-cell therapy of prostate cancer as we have previously shown that early stage HLA-A2⁺ prostate cancer patients have circulating T cells against both TARP₄₋₁₃ and TARP₂₇₋₃₅ (14). Until now, circulating T cells against TARP₄₋₁₃ in cancer patients was taken as indirect evidence that the TARP₄₋₁₃ peptide is in fact processed from the TARP

protein and presented correctly by HLA-A2 molecules to T cells. Herein, we show that the cloned TCR specifically recognizes the TARP₄₋₁₃ peptide on HLA-A2⁺ tumor cells transfected to express the full-length wild-type TARP protein, proving that the TARP₄₋₁₃ peptide is processed and presented. Importantly, we further show that TARP-TCR-engineered T cells can kill both prostate and breast cancer cell lines expressing the TARP antigen. The obtained data are taken as evidence that TARP₄₋₁₃ is a physiologically relevant T-cell target.

Because every T cell already has a unique TCR, genetic transfer of an exogenous TCR α and TCR β pair can lead to mispairing with endogenous TCR- α and TCR β chains. Mispairing gives rise to TCRs with unpredictable specificity and may create TCRs reactive with self-antigens and thereby generate autoreactive T cells. Furthermore, mispaired TCRs may compete for CD3 and thereby reduce the surface expression levels of the correctly paired transferred TCR. Several strategies have been used to avoid this from happening. Cohen et al. (18) successfully demonstrated replacement of the constant domain of the human TCR α and TCR β chains with the murine counterparts. Cohen et al. also reported that murinized receptors were overexpressed on the surface of human lymphocytes compared with their human counterparts and

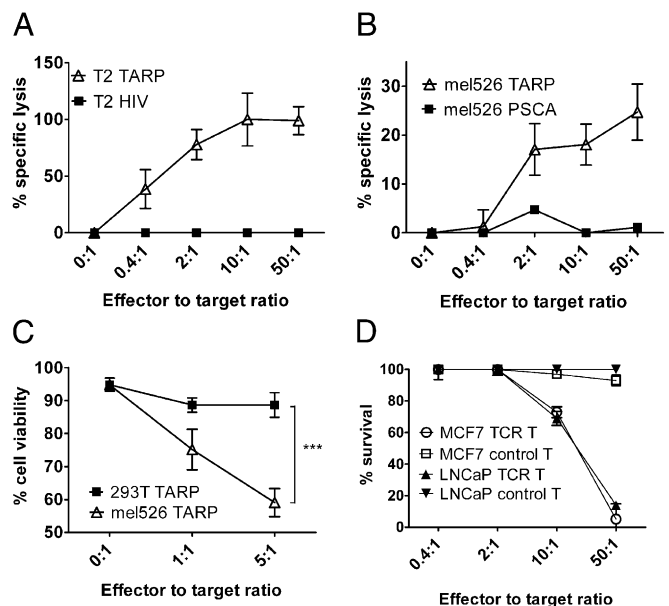


Fig. 4. TARP-TCR-modified T cells specifically kill TARP₄₋₁₃-presenting tumor cells. (A) TARP-TCR-engineered T cells were mixed with ⁵¹Cr-labeled T2 target cells, pulsed either with the HLA-A2-restricted TARP(P5L)₄₋₁₃ peptide (T2 TARP) or the irrelevant HLA-A2-restricted HIV-1_{pol}₄₇₆₋₄₈₄ peptide (T2 HIV). (B) TARP-TCR-engineered T cells were mixed with ⁵¹Cr-labeled mel526 target cells transfected with a plasmid encoding either the full-length wild-type TARP (mel526 TARP) or the irrelevant PSCA protein (mel526 PSCA). Specific killing was assayed in terms of ⁵¹Cr release from dead target cells after 4 h. Data represent triplicate samples from one representative experiment of two. (C) TARP-TCR-engineered T cells were mixed with either HLA-A2⁺ target cells (mel526) or HLA-A2⁻ target cells (HEK-293T) transfected with a plasmid encoding the full-length wild-type TARP protein. Target cell viability was assayed by propidium-iodide staining of CD3⁺ target cells after overnight coculture with TARP-TCR-engineered T cells. Asterisks denote significant difference (***) $P < 0.001$, two way ANOVA. Shown is pooled data from two experiments run in triplicates. (D) The HLA-A2-engineered prostate cancer cell line LNCaP and the IFN- γ pretreated breast cancer cell line MCF7 were transfected with a lentiviral vector encoding firefly luciferase. TARP-TCR T cells or nontransduced control T cells were cocultured for 72 h with luciferase-expressing LNCaP or MCF7 target cells at ratios 0.4:1, 2:1, 10:1, and 50:1. Luciferase expression was then examined as a measure of target cell viability. Luciferase expression from target cells not exposed to T cells was set to 100%.

were able to mediate higher levels of cytokine secretion when cocultured with peptide-pulsed antigen-presenting cells. Preferential pairing of murine constant regions and improved CD3 stability seemed to be responsible for these observations (18). We did not specifically address the issue of mispairing or compared human TCRs with murinized ones, but the finding that rapidly expanded TARP-TCR-engineered T cells have the same high-expression level of correctly formed TCR as before expansion, as shown by dextramer reactivity in Fig. 1B, strongly indicates that mispairing is rather low.

In conclusion, we present the cloning of a TCR directed against a physiologically relevant HLA-A2 epitope of an antigen that is highly and specifically expressed by normal prostate epithelial cells, prostate cancer cells, and breast cancer cells. T cells transduced with the TCR can specifically kill appropriate prostate and breast cancer cells and may therefore become important in future development of T therapy for prostate and breast cancer.

Materials and Methods

Cell Lines. The vector-packaging cell line HEK-293T (HLA-A2⁻), the breast cancer cell line MCF7 (HLA-A2⁺) and the melanoma cell line mel526 (HLA-A2⁺) were grown in DMEM, supplemented with 10% (vol/vol) FBS. The prostate cancer cell line LNCaP (HLA-A2⁺) was grown in RPMI-1640 supplemented with 10% (vol/vol) FBS, 10 mM HEPES, and 1 mM sodium pyruvate. LNCaP was modified to express relevant levels of HLA-A2 using a lentiviral vector (a kind gift from Richard Morgan, National Cancer Institute, Bethesda, MD). The antigen processing-deficient, HLA-A2⁺ T2 cell line was grown in RPMI-1640 supplemented with 10% FBS. Primary human peripheral blood mononuclear cells (PBMCs) were cultured in RPMI-1640 supplemented with 10% (vol/vol) human AB serum (our own production), 2 mM L-glutamine, 10 mM HEPES, 1% penicillin/streptomycin (PEST), and 20 μ M β -mercaptoethanol. T cells derived from PBMCs were cultured in PBMC medium with the addition of 100 IU/mL of IL-2 (proleukin; Novartis). All cell culture reagents were from Invitrogen except when stated otherwise. Cells were grown under standard cell culture conditions.

Generation of T-Cell Clones. Tetramer-sorted TARP₄₋₁₃-specific T cells (9) were plated in 96-well plates at a density of 0.6 cells per well in presence of 50 ng/mL anti-CD3 antibody (OKT-3; Nordic Biosite), 1,000 IU/mL IL-2, and 40,000 irradiated (50 Gy) feeder PBMCs (a pool from three donors). Growing clones were identified by visual inspection after 2 wk, transferred to larger wells, and restimulated in the same way. After an additional restimulation and 4 wk of culturing, reactive clones were identified by IFN- γ ELISA and TARP (P5L)₄₋₁₃/HLA-A2-dextramer staining (described below).

Isolation and Cloning of TCR Genes. To clone the T-cell receptor sequence from a TARP₄₋₁₃-specific T-cell, total RNA was isolated from IFN- γ /dextramer-positive clones using RNeasy Mini Kit (Qiagen). The TCR α and β chain sequences were obtained by 5' RACE PCR (SMARTer RACE cDNA Amplification Kit; Clontech) using the TCR α constant domain primer: 5'-GGT GAA TAG GCA GAC AGA CTT-3'; the TCR β constant domain primer: 5'-GTG GCC AGG CAC ACC AGT GT-3'; and the Advantage 2 polymerase mix (Clontech). The RACE PCR products were purified (PCR purification kit; Qiagen) tailed with 3' A (Taq polymerase; Invitrogen) and T/A-cloned into pCR2.1 (Invitrogen). Ten clones were sequenced and found to contain one unique TCR α chain and one unique TCR β chain. The coding sequence for the TARP₄₋₁₃-specific TCR α and TCR β chains was initiated with a Kozak sequence (19), codon optimized, and the two chains separated by the sequence for the T2A self-cleaving peptide (20). Furthermore, to improve pairing between the exogenous TCR α and β chains and reduce mispairing with endogenous human α - and β -chains, mouse constant domains replaced the human ones (18). The synthetic sequence was generated (Genscript) and we then subcloned it into a third generation self-inactivating lentiviral vector under the SFFV promoter to generate pBMN(TARP₄₋₁₃-TCR). The construct is illustrated in Fig. 1A.

Lentivirus Production. Vesicular stomatitis virus (VSV)-G pseudotyped lentiviral particles were produced in HEK-293T cells. Cells were transfected with pBMN(TARP₄₋₁₃-TCR) and the pLP1, pLP2 and pLP/VSFV (Invitrogen) packaging plasmids at a 1:1:1:2 ratio using polyethyleneimine (Sigma-Aldrich). Viral supernatant was collected 48 and 72 h posttransfection, filtered (0.45 μ m), and concentrated by ultracentrifugation at 75,000 \times g for 90 min at 4 $^{\circ}$ C using a Sorvall AH629 rotor. The viral pellet was resuspended in PBS and stored at -80 $^{\circ}$ C until further use. Lentiviral vectors expressing HLA-A2 and firefly luciferase were produced in the same fashion.

Generation of TARP-TCR-Modified T Cells. PBMCs were isolated from buffy coats of healthy blood donor using Ficol-Paque (GE Healthcare) and activated for 24–48 h with 100 ng/mL OKT-3 antibody and 100 IU/mL IL2. In some cases, CD8⁺ T cells were isolated using CD8⁺ T-cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. One million PBMCs or CD8⁺ T cells were transduced with 40 μ L of the concentrated TARP-TCR-coding lentivirus in the presence of 10 μ g/mL protamine sulfate (Sigma-Aldrich) and 100 IU/mL IL2 for 4 h. The transduction was repeated 24 h later. Transduced T cells were expanded using the rapid expansion protocol (REP) with OKT-3, IL2 and irradiated feeder cells, as previously described (21).

Flow Cytometry Analysis. To determine the percentage of TARP₄₋₁₃-specific CD8⁺ T cells after transduction, the cells were stained with a phycoerythrin (PE)-conjugated TARP(P5L)₄₋₁₃/HLA-A2-dextramer (Immudex), a FITC-conjugated anti-CD8 antibody (BioLegend) and an allophycocyanin (APC)-conjugated anti-CD3 antibody (BioLegend).

Peptides, Plasmids, and Preparation of Target Cells. The HLA-A2-restricted TARP(P5L)₄₋₁₃ (FLPSPLFFFL) and HIV-1p₁₄₇₆₋₄₈₄ (ILKEPVHGV) peptides were synthesized to a purity of >95% (Genscript). T2 target cells were pulsed with 50 μ g/mL TARP peptide or irrelevant HIV-1 peptide for 2 h in presence of 3 μ g/mL β 2 microglobulin (Sigma-Aldrich) and then used as target cells for evaluation of TARP-TCR-modified T cells. The full-length wild-type TARP-coding sequence was PCR amplified and cloned into pVAX1 (Invitrogen). As a negative control, the full-length PSCA coding sequence was PCR amplified and cloned into pShuttle (Stratagene). Mel526 cells were transfected with the TARP-encoding plasmid or the irrelevant PSCA-encoding plasmid using lipofectamine 2000 (Invitrogen). Twenty-four hours later, the cells were used as target cells for evaluation of TARP-TCR-engineered T cells.

ELISA. T cells (1×10^5) were mixed with peptide-pulsed T2 cells or plasmid-transfected mel526 cells at a 1:1 ratio and cocultured overnight. Supernatants were collected and IFN- γ ELISA was performed using an ELISA kit (Mabtech) according to the manufacturer's instructions.

T-Cell Proliferation Assay. TARP-TCR-modified T cells were labeled with 5 μ M CFSE (Invitrogen) for 10 min in PBS and the labeling was stopped by addition of medium containing 10% (vol/vol) human AB serum. The T cells were washed twice and 1×10^5 cells were cocultured with equivalent amount of target T2 cells pulsed with TARP peptide or irrelevant HIV-1 peptide. IL-2 (10 IU/mL) was added to the medium. T-cell proliferation, as detected as dilution of the CFSE dye for each cell division, was analyzed by flow cytometry 5 d later.

Flow Cytometry-Based CD107a Degranulation Assay. TARP-TCR-modified T cells (10^5 cells) were mixed with peptide-pulsed (TARP or HIV-1) T2 cells or plasmid-transfected (full-length TARP or PSCA) mel526 cells at a 1:1 ratio and cocultured overnight. For CD107a analysis the percentage of CD107a⁺ T cells were stained with a FITC-conjugated anti-CD107a antibody (BD Bioscience) followed by flow cytometry analysis.

⁵¹Cr Release Cytotoxicity Assay. T2 target cells (1×10^6) were labeled with 0.25 mCi ⁵¹Cr (Perkin-Elmer) for 1 h, washed five times, and pulsed with 50 μ g/mL peptide (TARP or HIV-1) for 2 h. Mel526 target cells, transfected with a plasmid encoding either the full-length TARP or PSCA were labeled with ⁵¹Cr for 1 h and washed five times. One-thousand target cells were cocultured with TARP-TCR-modified T cells at various effector to target cell ratios for 4 h in U-shaped 96 well microplates. Spontaneous release of ⁵¹Cr was assessed by the incubation of target cells in medium alone, and maximum release of ⁵¹Cr was determined by the incubation of target cells in 0.1% Triton X-100 (Sigma-Aldrich). Supernatants (100 μ L) containing the released ⁵¹Cr were collected and mixed with 75 μ L Optiphase SuperMix (Perkin-Elmer). Radioactivity was measured with a beta-counter (Wallac 1450 MicroBeta TriLux; Perkin-Elmer). Triplicate wells were averaged and the percentage of specific lysis was calculated as described previously (21). Spontaneous release was set to zero and in case that release from target cells was less than spontaneous release it was also set to zero.

Bioluminescent Luciferase Reporter Gene-Based Cell Killing Assay. LNCaP or MCF7 were modified to express luciferase using a lentiviral vector. MCF7 was treated with 1,000 IU/mL IFN- γ for 72 h before coculture with T cells. Ten-thousand target cells (LNCaP or MCF7) were plated in a 96-well plate and cocultured with transduced T cells in ratios of 0.4:1, 2:1, 10:1, or 50:1. After 72 h of coculture, the luciferase expression from viable target cells was

measured using Steady-Glo Luciferase Assay System (Promega), according to the manufacturer's instructions using a luminometer (Wallac Victor 2 Multilabel Counter; Perkin-Elmer). Luciferase activity from target cells not exposed to T cells was set as 100% cell viability (survival).

Flow Cytometry-Based Cytotoxicity Assay. TARP-TCR-modified T cells (10^5 cells) were mixed at a 1:1 ratio and cocultured overnight with mel526 (HLA-A2*) or HEK-293T (HLA-A2⁻) cells transfected with a plasmid encoding the full-length TARP protein. T cells were stained with the CD3 marker and excluded from the analysis. Dead target cells were discriminated from live ones by using propidium iodide (BD Biosciences).

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