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Targeting tumors using T-cell receptor gene transfer: a balance between efficacy and safety

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WT1-SPECIFIC TCRs DIRECTED AGAINST NEWLY IDENTIFIED PEPTIDES INSTALL ANTITUMOR REACTIVITY AGAINST ACUTE MYELOID LEUKEMIA AND OVARIAN CARCINOMA

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ABSTRACT

BACKGROUND

Transcription factor Wilms' tumor gene 1 (WT1) is an ideal tumor target based on its expression in a wide range of tumors, low-level expression in normal tissues and promoting role in cancer progression. In clinical trials, WT1 is targeted using peptide- or dendritic cell-based vaccines and T-cell receptor (TCR)-based therapies. Antitumor reactivities were reported, but T-cell reactivity is hampered by self-tolerance to WT1 and limited number of WT1 peptides, which were thus far selected based on HLA peptide binding algorithms.

METHODS

In this study, we have overcome both limitations by searching in the allogeneic T-cell repertoire of healthy donors for high-avidity WT1-specific T cells, specific for WT1 peptides derived from the HLA class I associated ligandome of primary leukemia and ovarian carcinoma samples.

RESULTS

Using broad panels of malignant cells and healthy cell subsets, T-cell clones were selected that demonstrated potent and specific anti-WT1 T-cell reactivity against five of the eight newly identified WT1 peptides. Notably T-cell clones for WT1 peptides previously used in clinical trials lacked reactivity against tumor cells, suggesting limited processing and presentation of these peptides. The TCR sequences of four T-cell clones were analyzed and TCR gene transfer into CD8+ T cells installed antitumor reactivity against WT1-expressing solid tumor cell lines, primary acute myeloid leukemia (AML) blasts, and ovarian carcinoma patient samples.

CONCLUSIONS

Our approach resulted in a set of naturally expressed WT1 peptides and four TCRs that are promising candidates for TCR gene transfer strategies in patients with WT1-expressing tumors, including AML and ovarian carcinoma.

INTRODUCTION

Transcription factor Wilms' tumor gene 1 (WT1) is expressed in a broad range of cancers, especially in ovarian carcinomas (OVCA), mesotheliomas, and acute myeloid leukemia (AML).^{1,2} These tumor types have low five-year survival rates of 49%, 12% and 29%, respectively, and there is a strong need for additional treatment options.^{3,4} In patients with OVCA and AML, WT1 expression is associated with poor prognosis.^{5,6} This corresponds to the promoting role of WT1 in cancer progression through the induction of tumor angiogenesis and metastasis formation.⁷ Amongst the numerous tumor associated antigens (TAA), WT1 is an ideal tumor target based on its expression in a wide range of hematological malignancies and solid tumors but low-level expression in normal tissues, as well as the tumor promoting characteristics. WT1 was even ranked by the US National Cancer Institute as the most promising tumor target for cancer vaccination.⁸ Currently, most clinical studies targeting WT1 use peptide- or dendritic cell-based vaccines. Mainly hematological malignancies are included in these trials, but also several solid tumor types such as gynecological malignancies and sarcomas are treated.⁹⁻¹¹ More recently, WT1 has been targeted by adoptive T-cell based therapies as well. In this rapidly growing therapy field promising clinical results have already been achieved in especially B-cell malignancies.¹²⁻¹⁴ For WT1, HLA class I restricted WT1-specific T cells were previously identified^{15,16}, and patients with AML and myelodysplastic syndrome (MDS) were treated with WT1-reactive CD8+ T cells or WT1-TCR engineered T cells (TCR-T cells) in clinical trials.¹⁷⁻¹⁹ In these clinical studies anti-leukemic reactivity and persistence of WT1-specific T cells have been reported. However, there are still many issues to be solved to improve avidity and increase killing potential of tumor cells by vaccine activated T cells or infused WT1-specific T-cell products.²⁰

WT1 is expressed during embryogenesis and involved in the embryonic development of kidneys, gonads, and several organs lined by the mesothelium. In adults, WT1 is involved in homeostasis processes for tissue maintenance and recovery, resulting in expression in renal podocytes, epicardial cells, Sertoli cells, granulosa cells and hematopoietic stem cells.²¹⁻²³ The expression levels in adults are expected to be sufficient to induce negative selection of high-avidity WT1-specific T cells during T-cell development, as mechanism to centrally delete self-reactive T cells. As a consequence, only the remaining low-avidity WT1-specific T cells present in the T-cell repertoire can be activated by WT1 peptide vaccines. In addition, the WT1-specific T cells and subsequently WT1-specific TCR-T cells transferred into patients were of low-avidity and demonstrated low antitumor reactivity.²⁴ Strategies to circumvent self-tolerance to WT1 have been developed. Increased reactivity was demonstrated by stimulating T cells with synthetic analog peptides derived from the WT1 protein^{25,26} or by using affinity enhanced WT1-specific TCRs.²⁷ Another strategy to circumvent self-tolerance is by searching for WT1-specific T cells in the allogeneic-HLA (allo-HLA) repertoire. Since allo-HLA reactivity of T cells is not subjected to negative selection, high-affinity allo-HLA-

restricted TCRs recognizing self-antigens are present in the TCR repertoire. This approach previously allowed the identification of high-affinity TCRs specific for several B-cell restricted antigens.²⁸⁻³⁰ Also for WT1, T cells recognizing HLA-A*02:01 restricted peptides were successfully isolated using this approach.^{16,31}

Besides the relative low-affinity of WT1-specific TCRs used in clinical studies, the selection of WT1-specific peptides used for peptide vaccination studies and identification of TCRs can most likely also be optimized. WT1 peptides were thus far selected based on HLA peptide binding algorithms, and although these peptides efficiently bind to HLA class I it is unknown whether these peptides are efficiently processed and presented in HLA class I at the cell surface of tumor cells. Furthermore, the thus far selected WT1 peptides were presented only in either HLA-A*02:01 or HLA-A*24:02, whereas more HLA alleles are necessary to cover a larger part of the patient population. In this study, WT1 peptides were identified from the HLA class I ligandome of primary leukemia and OVCA patient samples, and a large-scale search was performed to identify high-avidity WT1-specific CD8+ T cells targeting these peptides from the allo-HLA T-cell repertoire of healthy donors. Using broad panels of malignant cells and healthy cell subsets, we selected potent and specific WT1-reactive T-cell clones. The TCR sequences of these T-cell clones were analyzed and TCR gene transfer into CD8+ T cells installed antitumor reactivity against WT1-expressing solid tumor cell lines, as well as primary AML and ovarian carcinoma samples.

MATERIAL AND METHODS

WT1 EXPRESSION BY REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

WT1 expression was quantified by quantitative Polymerase Chain Reaction (qPCR). Total RNA was isolated using the RNAqueous-Micro Kit (Ambion) or ReliaPrep RNA Cell Miniprep System (Promega). First strand cDNA synthesis was performed with Moloney murine leukemia virus reverse transcriptase and Oligo (dT) primers (Invitrogen by Thermo Fisher Scientific). qPCR was performed using Fast Start TaqDNA Polymerase (Roche) and EvaGreen (Biotium), and gene expression was measured on the Lightcycler 480 (Roche). Expression was calculated as percentage relative to the average of housekeeping genes GUSB, VPS29 and PSMB4, which was set at 100%. All samples and genes were run in triplicate with 10 ng cDNA per reaction. The following primers were used: WT1 (forward: AGACCCACACCAG-GACTCAT, reverse: GATGCATGTTGTGATGGCGG), GUSB (forward: ACTGAACAGTCACCGACGAG, reverse: GGAACGCTGCACTTTTGGT), PSMB4 (forward: GTTCCGCAACATCTCTCGC, reverse: CATCAATCACCATCTGGCCG), VPS29 (forward: TGAGAGGAGACTTCGATGAGAATC, reverse: TCT-GCAACAGGGCTAAGCTG).

SAMPLE COLLECTION FOR PEPTIDE ELUTION

To identify T-cell epitopes derived from the WT1 gene, in total 37 tumor samples were collected. Cell pellets (2×10^9 – 610×10^9 cells) were made of 11 ALL samples, 15 AML samples, 1 HCL sample and 2 OVCA cell lines. Also 7 solid primary OVCA samples (2.3 gram – 30 gram) were included and 1 ascites primary OVCA sample (6×10^9 cells). The OVCA samples were residual material and collected anonymously. Solid OVCA tumors were sliced into small pieces and dead, clotted or non-tumor material was removed. The small tumor-pieces were added to a C-tube (Miltenyi Biotec) with ice cold buffer without detergent and cOmplete Protease Inhibitor (Sigma-Aldrich), to prevent protein degradation. Using a gentleMACS (Miltenyi Biotec) procedure the small tumor-pieces were dissociated until an almost homogenous cell solution. Benzonase (Merck) was added in a concentration of 125 IU/ml to remove DNA/RNA complexes during lysis. HLA typing was performed of all samples and WT1 expression was analyzed by qPCR.

HLA CLASS I-PEPTIDE ELUTION PROCEDURE, FRACTIONATION AND MASS SPECTROMETRY

Peptide elution was performed as outlined previously.³² In short, the cell pellets were lysed and subjected to an immunoaffinity column to collect bound peptide-HLA complexes, with either an HLA class-I antibody (W6/32, ATCC) or an HLA-A*02:01 antibody (BB7.2, ATCC). To separate the peptides, bound peptide-HLA complexes were dissociated with 10% acetic acid and filtrated using a 10 kDa membrane. Eluted peptide pools were either fractionated by strong cation exchange chromatography (SCX)³² or by high pH reversed phase fractionation (High pH-RP)³³. SCX and high pH-RP peptide fractions were lyophilized, dissolved in 95/3/0.1 water/ acetonitrile/formic acid v/v/v and subsequently analyzed by data-dependent MS/MS on either an LTQ FT Ultra equipped with a nanoflow liquid chromatography 1100 HPLC system (Agilent Technologies) or a Q Exactive mass spectrometer equipped with an easy-nLC 1000 (Thermo Fisher Scientific). Proteome Discoverer version 2.1 (Thermo Fisher Scientific) was used for peptide and protein identification, using the mascot search node for identification (mascot version 2.2.04) and the UniProt Homo Sapiens database (UP000005640; Jan 2015; 67,911 entries). All unique WT1-derived peptides with a length between 8 and 14 amino acids, a minimal Best Mascot Ion (BMI) score of 20, a mass accuracy of 10 ppm and predicted to bind to a common HLA molecule (HLA-A*01:01, -A*02:01, -A*03:01, -A*24:02, -B*07:02, -B*35:01, -C*07:01, -C*07:02) according to the netMHC peptide binding algorithm³⁴ were selected as candidate for peptide synthesis and validation.

PEPTIDE SYNTHESIS AND PMHC-MULTIMER PRODUCTION

Eight peptides met all the criteria and their synthetic peptides were in-house synthesized using standard Fmoc chemistry. By mass spectrometry the tandem mass spectra of the eluted peptides were validated with synthetic peptides (Figure S1). Of in total 12 WT1-derived peptides, pMHC-multimer complexes were generated with minor modifications.³⁵ In short,

monomers consisting of the selected HLA allele heavy chain, human beta-2 microglobulin (B2M) light chain and selected peptide were purified by gel-filtration high-performance liquid chromatography and biotinylated. Subsequently, pMHC-multimers were generated by adding PE-conjugated streptavidin (Invitrogen, Thermo Fisher Scientific).

CELL CULTURE

T cells were cultured in T-cell medium (TCM) composed of Iscove's Modified Dulbecco's Medium (IMDM) (Lonza), 5% heat-inactivated Fetal Bovine Serum (FBS) (Gibco, Thermo Fisher Scientific), 5% human serum (Sanquin Reagents), 1.5% 200 mM L-glutamine (Lonza), 1% 10,000 U/mL penicillin/streptomycin (Pen/Strep; Lonza) and 100 IU/mL IL-2 (Novartis Pharma). Every 10-14 days, 0.2×10^6 T cells were (re)stimulated with 1×10^6 irradiated (35 Gy) PBMCs, 0.1×10^6 irradiated (55 Gy) EBV-LCLs and 0.8 $\mu\text{g}/\text{mL}$ phytohemagglutinin (PHA) (Oxoid Microbiology Products, Thermo Fisher Scientific).

Most tumor cell lines were cultured in IMDM, 10% FBS, 1.5% L-glutamine and 1% Pen/Strep. OVCA cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose 4.5 g/L, NEAA) (Gibco), 8% FBS, 2% 200 mM L-glutamine and 1% 10,000 U/mL Pen/Strep. Using the Plasmotest Mycoplasma Detection Kit (InvivoGen), all cell lines were found to be mycoplasma negative. Of all cell lines, HLA typing was performed and WT1 expression was analyzed by qPCR. If needed, the WT1 gene or an HLA allele was introduced by retroviral transduction. These genes were expressed in MP71 retroviral backbone vectors with marker genes nerve growth factor receptor (NGF-R), green fluorescent protein (GFP), CD34 or mouse CD19 (mCD19). Target cells were enriched for marker gene expression via magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) and purity was confirmed by FACS.

Primary AML samples were thawed one day before being used as target cells in screening experiments. Cells were cultured overnight at 37 degrees and 5% CO₂ in IMDM containing 10% human serum and if needed, live cells were isolated using Ficoll gradient separation. Blast percentage of the AML samples was on average 83% (range 40% – 99%), as determined by FACS expression (CD13, CD33 and CD34). Primary OVCA cells were thawed three days before being used as target cells in screening experiments. Cells were cultured in IMDM, 10% FBS, 1.5% 200 mM L-glutamine and 1% 10,000 U/mL Pen/Strep, on FBS pre-coated plates.

HEALTHY CELL SUBSET ISOLATION

Hematopoietic healthy cell subsets were isolated from PBMCs of healthy donors, either HLA-A*01:01 and HLA-B*35:01 positive or HLA-A*02:01 positive. CD14, CD19 and CD34 positive cells were enriched by MACS using anti-CD14 MicroBeads (Miltenyi Biotec/200-070-118), anti-CD19 MicroBeads (Miltenyi Biotec/130-060-301) or anti-CD34 MicroBeads (Miltenyi Biotec/130-046-702). Activated CD19+ B-cells were generated by

co-culturing CD19+ cells on CD40L-transduced irradiated (70 Gy) mouse fibroblasts for 7 days in IMDM supplemented with 2 ng/mL IL-4 (Schering-Plough) and 10% human serum. Immature and mature CD14-derived dendritic cells (DCs) were differentiated *in vitro* from isolated CD14+ cells. Immature DCs were generated by culturing 0.1×10^6 cells/mL for 3 days in IMDM supplemented with 100 ng/mL GM-CSF (Sandoz Novartis Pharma), 500 IU/mL IL-4 (Schering-Plough), and 10% human serum. Mature DCs were generated by culturing immature DCs for an additional 3 days in IMDM supplemented with 100 ng/mL GM-CSF, 10 ng/ml TNFalpha (CellGenix), 10 ng/mL IL-1b (Bioscience Invitrogen), 10 ng/mL IL-6 (Sandoz Novartis Pharma), 1 µg/ml PGE-2 (Sigma Aldrich), 500 IU/mL IFN-γ (Boehringer Ingelheim), and 10% human serum. Isolated CD34+ hematopoietic precursor cells were directly used after isolation. Purity of the isolated and generated cells was assessed using FACS analysis. Fibroblasts from skin biopsies were cultured in DMEM with 1g/L glucose (Lonza BE12-708F) and 10% FBS. Keratinocytes from skin biopsies were cultured in keratinocyte serum free medium (Thermo Fisher Scientific 17005-059) supplemented with 30 µg/mL bovine pituitary extract (BPE) and 2 ng/mL epithelial growth factor (EGF) (both Thermo Fisher Scientific 37000-015).

ANTIBODIES AND FACS ANALYSIS

FACS was performed on an LSR II flow cytometer (BD Biosciences) and data was analyzed using FlowJo software (TreeStar). T cells were stained with the following conjugated antibodies: CD4 FITC (BD/555346), CD14 FITC (BD/555397), CD19 FITC (BD/555412), CD8 AF700 (Invitrogen/MHCD0829), murine TCR-β (mTCR-β) APC (BD/553174) and pMHC-multimers PE. Target cells with transduced WT1 or HLA-alleles were stained with: NGFR/CD271 APC (Sanbio/CL10013APC), CD34 APC (BD/555824), murine CD19 PE (BD/557399), HLA-A2 PE (BD/558570). Non-malignant hematopoietic subsets with: CD14 FITC (BD/555397), CD19 FITC (BD/555412), CD34 APC (BD/555824), CD80 PE (BD/557227) and CD86 PE (BD/555658). AML samples with: CD13 PE (BD/347406), CD33 FITC (BD/555626) and CD34 APC (BD/555824).

ISOLATION OF WT1-SPECIFIC T CELLS BY PMHC-MULTIMER ENRICHMENT

Buffy coats of healthy donors negative for HLA-A*01:01, HLA-A*02:01, HLA-A*03:01, HLA-A*24:02, or HLA-B*35:01 were collected after informed consent (Sanquin). PBMCs were isolated using Ficoll gradient separation and incubated with a selection of WT1-specific pMHC-multimers for 1 hour at 4°C or 15 minutes at 37°C. pMHC-multimers were only included if the healthy donor was negative for the restricted HLA allele. pMHC-multimer bound cells were MACS enriched using anti-PE MicroBeads (Miltenyi Biotec/130-048-801). The positive fraction was stained with AF700-conjugated antibody against CD8 and FITC-conjugated antibodies against CD4, CD14 and CD19. pMHC-multimer and CD8 positive cells were single-cell sorted using an Aria III cell sorter (BD Biosciences) in a 96 well round bottom plate containing 5×10^4 irradiated PBMCs (35Gy) and 5×10^3 EBV-JY cells (55Gy) in 100ul T-cell medium with 0.8 µg/ml PHA. T-cell recognition was assessed 10 – 14 days after

stimulation, followed by restimulation or storage of the selected T-cell clones.

T-CELL REACTIVITY ASSAYS

T-cell recognition was measured by an IFN- γ ELISA (Sanquin). 5,000 T cells were cocultured overnight with target cells in various effector-to-target (E:T) ratios in 60 μ l TCM in 384-well flat-bottom plates (Greiner Bio-One). To upregulate HLA expression, all adherent target cells were treated for 48 hours with 100 IU/ml IFN- γ (Boehringer Ingelheim) before coculture. All T cells and target cells were washed thoroughly before coculture to remove expansion-related cytokines. Supernatants were transferred during the ELISA procedure using the Hamilton Microlab STAR Liquid Handling System (Hamilton company) and diluted 1:5, 1:25 and/or 1:125 to quantify IFN- γ production levels within the area of the standard curve. The Hamilton System was also used to split 96-well T-cell cultures into 4 wells of 384-well flat bottom plates during our large-scale T-cell search in 28 healthy donors, making it feasible to screen the T cells against different combinations of Raji cells loaded with and without peptides (1 μ M).

T-cell mediated cytotoxicity was measured in a 6-hour 51 chromium release assay. Target cells were labeled with 100 μ Ci 51 chromium (PerkinElmer) for 1 hour at 37°C, washed, and co-cultured with T cells at various E:T ratios in 100 μ l TCM per well in 96-well U-bottom culture plates (Costar). Spontaneous and maximum 51 chromium release for all targets were measured in separate plates with per well 100 μ l TCM or 100 μ l TCM with 1% Triton-X 100 (Sigma-Aldrich), respectively. After 6 hours of coculture, 25 μ l supernatant was harvested, transferred to 96-well LumaPlates (PerkinElmer) and 51 chromium release was measured in counts per minute on a 2450 Microbeta² plate counter (PerkinElmer). The percentage of killed target cells was calculated with the following formula = ((experimental release – spontaneous release)/(maximum release – spontaneous release)) *100.

TCR IDENTIFICATION AND PRODUCTION OF RETROVIRAL SUPERNATANTS

TCR α and β chains of the selected T-cell clones were identified by sequencing with minor modifications, as previously described.³⁶ mRNA was isolated by the Dynabeads mRNA DIRECT Kit (Invitrogen) or total RNA was isolated by the ReliaPrep RNA cell Miniprep System (Promega). TCR cDNA was generated using TCR constant α and β primers, a SA.rt anchor template-switching oligonucleotide (TSO), and SMARTScribe Reverse Transcriptase (Takara, Clontech).³⁷ The TCR α and β products were generated in a first PCR using Phusion Flash (Thermo Fisher Scientific), followed by a second PCR that was used to include 2-sided barcode sequences for the different T-cell clones. Barcoded TCR PCR products were pooled and TCR sequences were identified by HiSeq or NovaSeq (GenomeScan). The V α and V β families were determined of the NGS data using the MiXCR software and ImMunoGeneTics (IMGT) database.³⁸ The TCR chains were codon optimized, synthesized and cloned in MP71-TCR-flex retroviral vectors by Baseclear. The MP71-TCR-flex vector contains codon-optimized and cysteine-modified murine TCR $\alpha\beta$ constant domains and P2A sequence to link TCR

chains, resulting in optimized TCR expression and increased preferential pairing.³⁹ Apart from the WT1-specific TCRs, a murinized CMV-specific TCR (NLVPMVATV peptide presented in HLA-A*02:01) was included as a negative control. Phoenix-AMPHO (ATCC) cells were transiently transfected with the created constructs and after 48 hours retroviral supernatants were harvested and stored at -80°C.

TCR GENE TRANSFER TO HEALTHY DONOR CD8+ T CELLS

CD8+ T cells were isolated from PBMCs of different healthy individuals/donors by MACS using anti-CD8 MicroBeads (Miltenyi Biotec/130-045-201). CD8+ T cells were stimulated with irradiated autologous feeders (40 Gy) and 0.8 µg/mL PHA in 24-well flat-bottom culture plates (Costar). Two days after stimulation, CD8+ T cells were transferred to 24-well flat-bottom suspension culture plates (Greiner Bio-One) for retroviral transduction. These plates were first coated with 30 µg/ml retronectin (Takara, Clontech) and blocked with 2% human serum albumin. Retroviral supernatants were added, and plates were centrifuged at 3000 g for 20 minutes at 4°C. After removal of the retroviral supernatant, 0.3×10^6 CD8+ T cells were transferred per well. After O/N incubation, CD8+ T cells were transferred to 24-well flat-bottom culture plates (Costar). Seven days after stimulation, CD8+ T cells were MACS enriched for the murine TCR, using mTCR-β APC antibody (BD/553174) and anti-APC MicroBeads (Miltenyi Biotec/130-090-855). Ten days after stimulation, CD8+ T cells were functionally tested and purity was checked by FACS.

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism software (Version 9.0.1.). The statistical test used is indicated in the figure legend, $P < 0.05$ was considered significant. Significance levels are indicated as $p < .05$ *, $p < .01$ **, $p < .001$ ***, and $p < .0001$ ****.

STUDY APPROVAL

AML patient samples were used from the Leiden University Medical Center (LUMC) Biobank for Hematological Diseases. This study was approved by the Institutional Review Board of the LUMC (approval number 3.4205/010/FB/jr) and the METC-LDD (approval number HEM 008/SH/sh). Materials from patients and healthy individuals were collected after written informed consent according to the Declaration of Helsinki. The OVCA samples were residual material and collected anonymously.

RESULTS

IDENTIFICATION OF WT1-DERIVED PEPTIDES IN OVARIAN CARCINOMA AND LEUKEMIA SAMPLES

To identify WT1-derived peptides that are efficiently expressed in HLA class I at the cell surface of tumors, patient samples were selected based on WT1 expression levels. By qPCR, gene expression was measured in primary OVCA, acute lymphoblastic leukemia (ALL), and AML samples, and healthy cell subsets. WT1 expression ($\geq 5\%$ relative expression compared to housekeeping genes) was observed in all OVCA samples (16/16), most ALL samples (3/4) and in more than half of the AML samples (18/30) (Figure 1A). In addition, WT1 was widely expressed across a variety of tumor cell lines and, although not significant, on average the highest WT1 expression was observed in OVCA, ALL and AML cell lines (Figure 1B). These results indicate that tumor samples of patients with OVCA, ALL and AML could be used for the identification of naturally expressed WT1 peptides.

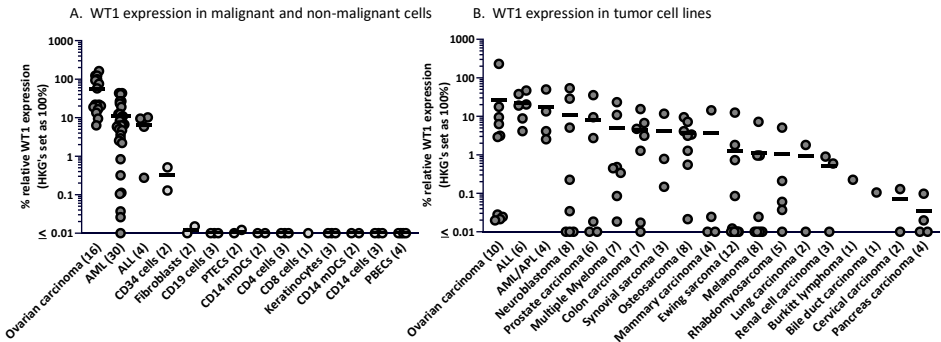


Figure 1. High WT1 expression in primary OVCA, ALL, and AML samples, low or absent expression in various healthy cell subsets

WT1 mRNA expression was measured by qPCR. WT1 expression is shown as percentage relative to the three housekeeping genes GUSB, VPS29 and PSMB4, which was set at 100%. The minimum gene expression is set at 0.01%. Dots illustrate in (A) primary tumor samples, or healthy cell subsets, and in (B) tumor cell lines. The vertical bars indicate mean and number of samples/cell lines per group are shown between brackets. Differences were tested by a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. The WT1 expression levels in the primary tumor samples in A were significantly different compared to the healthy cell subsets combined ($p < 0.043$), no significant differences were observed between any of the tumor cell line types in B. ALL: acute lymphoblastic leukemia, AML: acute myeloid leukemia, APL: acute promyelocytic leukemia, iDCs and mDCs: immature and mature dendritic cells, OVCA: primary ovarian carcinoma, PBECs: primary bronchus epithelial cells, PTECs: proximal tubular epithelial cells.

The HLA class I associated ligandome was determined of 11 patients with ALL, 15 patients with AML, 1 patient with hairy cell leukemia (HCL), and 8 patients with OVCA, as well as 2 OVCA cell lines. All WT1 peptides with a length between 8 and 14 amino acids, a minimal Best Mascot

Ion (BMI) score of 20 and a mass accuracy of 10 ppm were selected as candidate for peptide synthesis and validation. Peptides were synthesized if they were predicted to bind common HLA molecules (HLA-A*01:01, -A*02:01, -A*03:01, -A*24:02, -B*07:02, -B*35:01, -C*07:01, -C*07:02) according to netMHC peptide binding algorithm³⁴, and matched the HLA typing of the material from which the peptides originated (Table S1). Eight peptides were identified and the sequences of these peptides were validated by comparing mass spectra of eluted peptides with the mass spectra of synthetic peptides (Figure S1). WT1 peptides frequently studied and used in clinical trials; RMFPNAPYL (HLA-A*02:01 restricted)¹⁹, CMTWNQMNL (HLA-A*02:01 and HLA-A*24:02 restricted)^{17,31} and RWPSCQKKF (HLA-A*24:02 restricted)⁴⁰ were not identified in our large HLA-ligandome database, whereas 20 and 9 of the eluted tumor samples expressed HLA-A*02:01 or HLA-A*24:02, respectively. Considering the frequent use of these peptides, we added them to our final set of WT1 peptides, and in total 12 PE-labeled peptide-MHC (pMHC)-multimer complexes were generated (Table 1).

Table 1. Overview of WT1 peptides included in this study

Nr	Peptide	HLA	Sample/cell line source	BMI
1	WTEGQSNHSTGY	A*01:01	OVCA-G1, ALL-1833, AML-4443, AML-10197	43
2	VLDFAAPPASAY	A*01:01	AML-4443	36
3	ALLPAVPSL	A*02:01	OVCA-L23	31
4	VLDFAAPPGA	A*02:01	OVCA-L23	26
5	FGPPPPSQA	A*02:01	OVCA-L23, AML-10197	42
6	AQFPNHFSK	A*03:01	Cell line-COV362.4	20
7	HAAQFPNHSF	B*35:01	HCL-4512	36
8	TPYSSDNLY	B*35:01	ALL-2184	41
9	RMFPNAPYL	A*02:01	x	x
10	CMTWNQMNL	A*02:01	x	x
11	RWPSCQKKF	A*24:02	x	x
12	CMTWNQMNL	A*24:02	x	x

Overview of the 12 WT1 peptides included in this study. For the eight WT1 peptides identified in our HLA ligandome analyses, the source and BMI is listed. The four peptides not identified in this study, but previously described in literature, were added at the end of the table. ALL: acute lymphoblastic leukemia, AML: acute myeloid leukemia, BMI: best Mascot ion score, HCL: hairy cell leukemia, OVCA: primary ovarian carcinoma.

LARGE-SCALE WT1-REACTIVE T-CELL SEARCH IN THE ALLO-HLA T-CELL REPERTOIRE OF 28 HEALTHY DONORS

With the pMHC-multimers we searched for WT1-reactive T cells, with the ultimate goal to identify high-affinity WT1-specific TCRs. PBMCs of 28 healthy HLA typed donors were incubated with pMHC-multimers, pMHC-multimer positive CD8+ T cells were enriched by MACS, and single-cell sorted (Table S2). pMHC-multimers were only included if the donor was negative for the HLA allele, enabling us to search within the allo-HLA T-cell repertoire, and thereby circumventing self-tolerance. On average 650×10^6 PBMCs per donor were used and

between 20 and 658 pMHC-multimer positive CD8+ T-cell clones were expanded after single-cell sorting (Table S2). Two weeks after clonal expansion, a peptide specificity screening was conducted on 7916 isolated T-cell clones. Burkitt lymphoma cell line Raji, negative for WT1, transduced with HLA-alleles of interest, were pulsed with the WT1 peptide pool, and used as stimulator cells. In total 461 T-cell clones (6%) were WT1 peptide specific, recognizing WT1 peptide pool pulsed Raji cells, whereas non-pulsed Raji cells were not recognized. The other T-cell clones were either reactive against all stimulator cells, nonreactive or reactive against one specific HLA-allele, independent of added peptides. The peptide-specific T-cell clones were subsequently tested against the individual peptides and screened for recognition of endogenously processed and presented WT1. Of the 461 T-cell clones, 71 (15%) clones recognized WT1 transduced Raji cells. The peptide specificity of these T-cell clones is summarized in Table S3, T-cell clones were identified for 9 out of 12 WT1 peptides.

IDENTIFICATION OF FIVE POTENT AND SPECIFIC WT1-REACTIVE T-CELL CLONES

To select the most potent and specific WT1-reactive T-cell clones, the 71 T-cell clones were analyzed in additional screenings. This selection approach is illustrated in Figure S2 for seven T-cell clones recognizing the VLDFAPPGA peptide in HLA-A*02:01. T cells were tested against a tumor cell line panel composed of WT1 positive and negative tumor cell lines that were positive or transduced with the corresponding HLA restriction molecule (Figure S2A). The T-cell clones recognizing at least two WT1 positive tumor cell lines, combined with no recognition of WT1 negative tumor cell lines were selected (28/71 T-cell clones). To avoid allo-HLA cross-reactivity, selected T-cell clones were additionally screened against a panel of 25 Epstein-Barr virus transformed lymphoblastoid cell lines (EBV-LCL), expressing all HLA alleles with an allele frequency > 1% present in the Caucasian population (Figure S2B). Eight T-cell clones were excluded that showed HLA cross-reactivity against one or several prevalent HLA alleles, exemplified by clone 23.2E4. Those T-cell clones that recognize only one non-prevalent HLA allele were not excluded, exemplified by clone 20.3D10. Finally, to investigate clinical potential of the remaining 20/71 T-cell clones, we analyzed the antitumor reactivity against primary AML and OVCA patient samples expressing variable levels of WT1 (Figure S2C).

Using this selection strategy we identified 5/71 promising WT1-specific T-cell clones, that demonstrated the most potent antitumor reactivity against WT1 positive primary AML and/or OVCA patient samples. The recognition patterns of these T-cell clones, recognizing five different WT1 peptides in the context of three different HLA class I molecules, are shown in Figure 2. Clone 20.3D10 is specific for VLDFAPPGA in HLA-A*02:01 (VLD/A2), clone 22.1H1 is specific for ALLPAVPSL also in HLA-A*02:01 (ALL/A2), clone 12.5H9 is specific for VLDFAPPGASAY in HLA-A*01:01 (VLD/A1), clone 17.2G4 is specific for TPYSSDNLY in HLA-B*35:01 (TPY/B35), and clone 17.2D6 is specific for HAAQFPNHSF also in HLA-B*35:01 (HAA/B35). All five WT1-specific clones demonstrated antitumor reactivity against WT1 positive tumor cell lines. Only those tumor cell lines with a WT1 expression below 15% were

more variable recognized (Figure 2A). As depicted in Figure 2B, two of the five T-cell clones showed HLA cross-reactivity against one non-prevalent HLA allele. The global frequencies of these HLA-alleles are low (HLA-A*33:01: 1.77% and HLA-A*02:05: 1.17%), implicating that when one of these TCRs would be used for clinical practice only a small group of patients would not be suitable candidates for this particular TCR-based therapy⁴¹. Apart from the recognized EBV-LCLs, no other target cells used in the assays expressed these HLA-alleles. Furthermore, primary AML and OVCA patient samples with WT1 expression were recognized by the different WT1-specific T-cell clones (Figure 2C). Interestingly, not all recognition patterns correlated with WT1 expression levels. For example clone 12.5H9^{VLD/A1} showed the highest recognition of AML-4443 (13% relative WT1 expression), from which the VLD/A1 peptide was eluted (Table 1), whereas the other AML samples with WT1 expression between 23-27% were less well recognized. In addition, clone 20.3D10^{VLD/A2} and the other VLD/A2 reactive T-cell clones (Figure S2) did not recognize AML-6588 (10%) and AML-4716 (3%), while the 22.1H1^{ALL/A2} clone recognized both. This also accounts for some of the HLA-B*35:01 positive primary AML patient samples. Clone 17.2G4^{TPV/B35} recognized AML-5905 (44%), whereas clone 17.2D6^{HAA/B35} did not, while both recognized the other two primary AML patient samples. This could not be explained by differences in antigen presenting capacity of the primary AML patient samples, since similar reactivity by allo-HLA reactive T-cell clones directed against these different AML samples was observed (Figure S3A-B).

Notably none of the selected T-cell clones were specific for the peptides that were previously identified based on HLA peptide binding algorithms, including the most frequently used RMFPNAPYL peptide presented in HLA-A*02:01 (RMF/A2). Although high numbers of T-cell clones specific for RMF/A2 peptide were identified, of which a limited number were reactive against overexpressed WT1 transduced Raji cells (546% relative WT1 expression), all these T-cell clones were not reactive against WT1 positive tumor cell lines and primary AML patient samples. To illustrate the limited reactivity, RMF/A2 and VLD/A2 reactive T-cell clones isolated from five donors were compared. In Figure 3A it is shown that 12% of the RMF/A2-specific T-cell clones were reactive against WT1 transduced cells, whereas over 33% of the VLD/A2-specific T-cell clones were reactive against WT1 transduced cells. The most potent T-cell clones for both peptides were selected and no difference in peptide sensitivity was observed in a peptide titration (Figure 3B). However, reactivity against WT1 positive tumor cell lines with 19% to 230% relative WT1 expression was very limited by all RMF/A2 clones (Figure 3C), and none of the primary AML patient samples with 6% to 44% relative WT1 expression were recognized (Figure 3D). In contrast, VLD/A2-specific T-cell clones were highly reactive against these WT1 positive tumor cell lines as well as primary AML patient samples (Figure 3C-D).

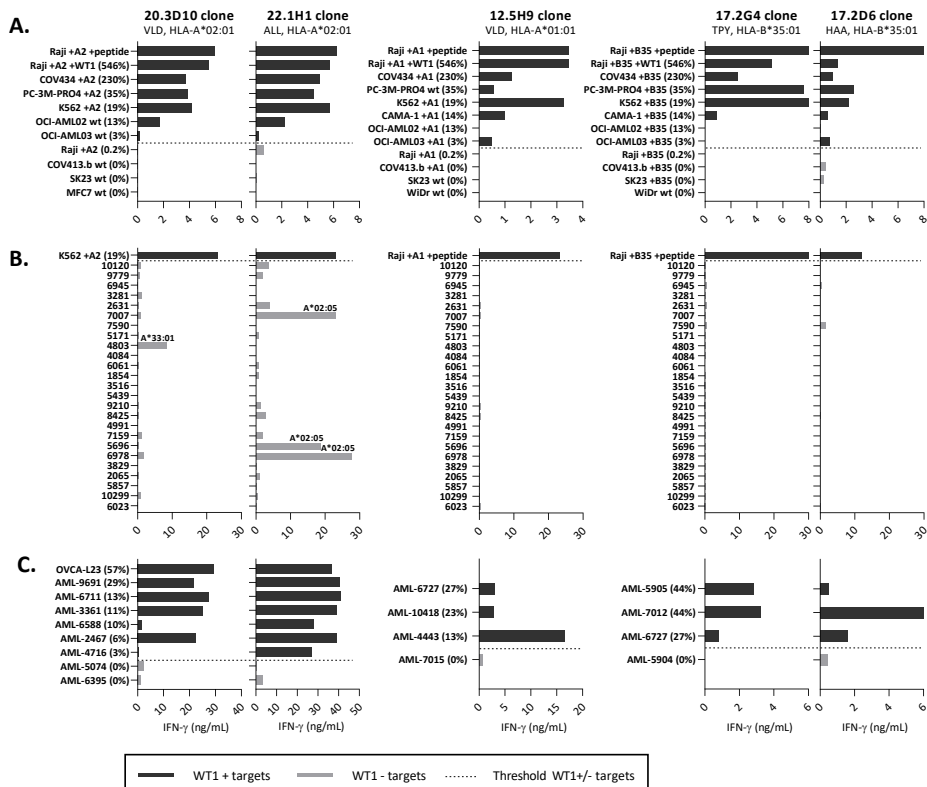


Figure 2. Recognition patterns of the five most promising WT1-specific T-cell clones

Recognition patterns based on IFN- γ production (ng/mL) after overnight coculture assays with three screening panels. **(A)** Panel with WT1+ and WT- tumor cell lines (E:T = 1:6). **(B)** Panel with 25 EBV-LCLs, expressing all frequent HLA alleles (with an allele frequency $\geq 1\%$) present in the Caucasian population (E:T = 1:6). The HLA-allele is depicted if only one HLA-allele is recognized by the T-cell clone, meeting the requirement that all EBV-LCLs with this HLA-allele are recognized. **(C)** Panel with primary AML samples (E:T = 1:16) and OVCA patient samples (E:T = 1:6). Blast percentage of the AML samples was on average 83% (range 40% – 99%), as determined by FACS expression (CD13, CD33 and CD34). All cell lines in panel A and C express the HLA-allele that presents the targeted peptide, either wildtype (wt) or the HLA-allele was introduced by transduction (+A2, +A1, +B35). Percentage relative WT1 expression is depicted, as determined by qPCR. Dark grey bars depict high WT1+ targets and light grey bars the WT1- targets. Bars represent averaged duplicate values and are representative of two independent experiments. AML: acute myeloid leukemia, EBV-LCL: Epstein-Barr virus transformed lymphoblastoid cell lines, FACS: fluorescence-activated cell sorting, OVCA: primary ovarian carcinoma.

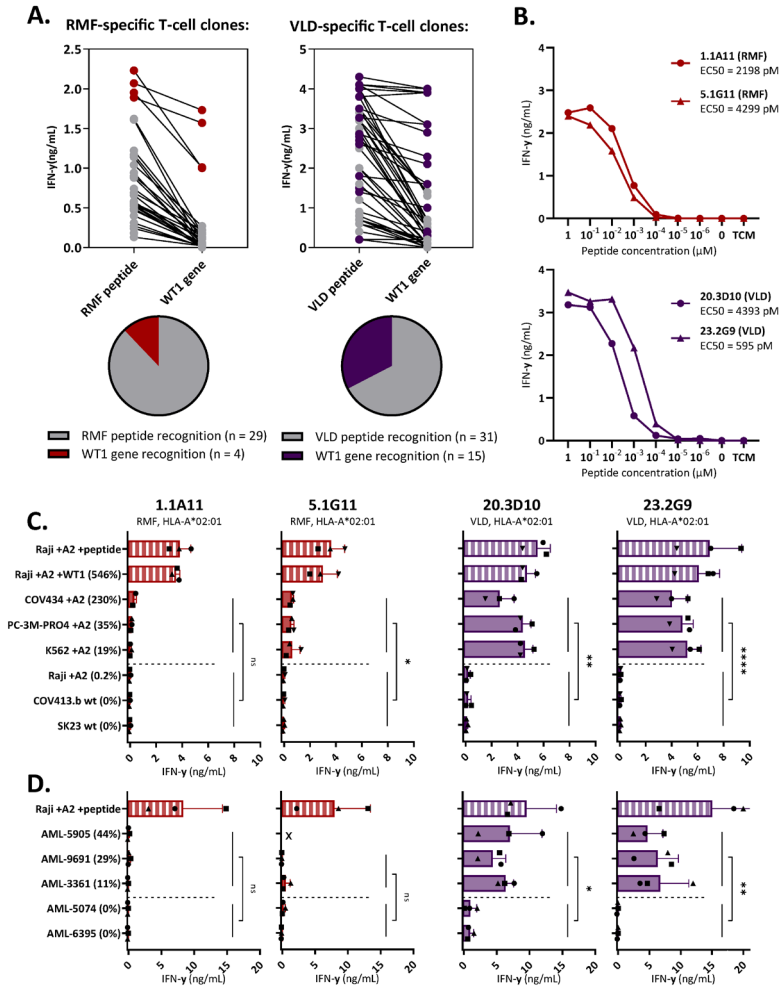


Figure 3. Limited WT1-specific reactivity of the RMF-specific T-cell clones

Comparison of T-cell clones recognizing the RMFPNAPYL (RMF) and VLDFAPPGA (VLD) peptide, both presented in HLA-A*02:01. **(A)** IFN- γ production (ng/mL) of T-cell clones recognizing Raji pulsed with RMF/A2 or VLD/A2 peptide (1 μ M) identified in five healthy donors. T-cell clones also recognizing Raji transduced with WT1 (WT1 gene) on a similar level (>50%), are depicted with colored dots. **(B)** The four most potent T-cell clones stimulated overnight with Raji pulsed with titrated peptide. EC50 values represent the peptide concentrations needed to induce 50% of the maximum cytokine production, values were calculated based on sigmoidal curves. **(C)** T-cell clones stimulated overnight with tumor cell lines (E:T = 1:6). All cell lines express the HLA-A*02:01, either wildtype (wt) or introduced by transduction (+A2). Percentage relative WT1 expression is depicted, as determined by qPCR. **(D)** T-cell clones stimulated overnight with primary AML patient samples (E:T = 1:16). (x = Recognition of AML-5905 is not shown for clone 5.1G11, since this sample is HLA-B*35:01 positive and the 5.1G11 clone demonstrated allo-HLA reactivity against this allele in an EBV-LCL panel). **(C-D)** Symbols represent averaged duplicate values from three independent experiments. Mean and standard deviation (SD) are depicted. WT1+ and WT1- targets were grouped, and reactivity of WT1 T-cell clones directed against these groups were compared using an unpaired t-test (two-sided). AML: acute myeloid leukemia, EBV-LCL: Epstein-Barr virus transformed lymphoblastoid cell lines, ns: not significant.

TCR GENE TRANSFER IN CD8+ T CELLS INSTALLS POTENT WT1-SPECIFIC REACTIVITY WITHOUT ON-TARGET OFF-TUMOR TOXICITY

Next, to test whether the TCRs of the selected T-cell clones can be used for TCR gene therapeutic strategies, we analyzed their TCRs in more detail. We continued with four of the five T-cell clones: 20.3D10^{VLD/A2}, 22.1H1^{ALL/A2}, 12.5H9^{VLD/A1}, and 17.2G4^{TPV/B35} based on potency. The TCR α and β chains were identified by sequencing and transferred using retroviral vectors into CD8+ T cells of four different donors. TCR-engineered T cells (TCR-T cells) were enriched based on murine TCR (mTCR) expression and pMHC-multimer staining demonstrated that the TCR-T cells efficiently express the TCR at the cell surface (Figure S4A). Functional reactivity of the TCR-T cells measured by peptide titration experiments was quite comparable to their parental T-cell clones (Figure S4B).

To investigate the antitumor potential of the WT1 TCR-T cells, they were screened against multiple tumor panels, assessed for IFN- γ production and compared with CMV TCR-T cells as negative control. The four WT1 TCRs demonstrated effective antitumor reactivity, recognizing WT1 positive tumor cell lines, whereas WT1 negative cell lines were not recognized (Figure 4A). The included WT1 positive primary AML and OVCA patient samples were recognized (Figure 4B-C). The level of recognition of these primary tumor samples correlated with the level of WT1 expression, except for TCR-T 12.5H9^{VLD/A1} that only recognized the AML-4443 sample in which the VLD/A1 peptide was initially eluted (Table 1).

To investigate the safety profile of the TCR-T cells, the engineered T cells were tested against a variety of different healthy cell subsets. Keratinocytes, fibroblasts, and several hematopoietic cell subsets, including CD34+ hematopoietic precursor cells, CD14+ derived immature and mature DCs, and B cells were tested for recognition by the TCR-T cells. No WT1 expression was detected in these healthy cell subsets, only in CD34+ hematopoietic precursor cells limited WT1 expression (<0.5%) was observed (Figure 1A). The TCR-T cells demonstrated no reactivity against the different healthy cell subsets, even CD34+ hematopoietic precursor cells were not or very limited (20.3D10^{VLD/A2} TCR) recognized, indicating that these TCR-T cells do not exhibit on-target off-tumor reactivity (Figure 4D). Reactivity against all targets was observed by the allo-HLA reactive T-cell clones (Figure S3C-F). In conclusion, TCR gene transfer of WT1-TCRs into CD8+ T cells installed high WT1-specific reactivity against WT1 positive tumor cells, without indications of on-target off-tumor reactivity.

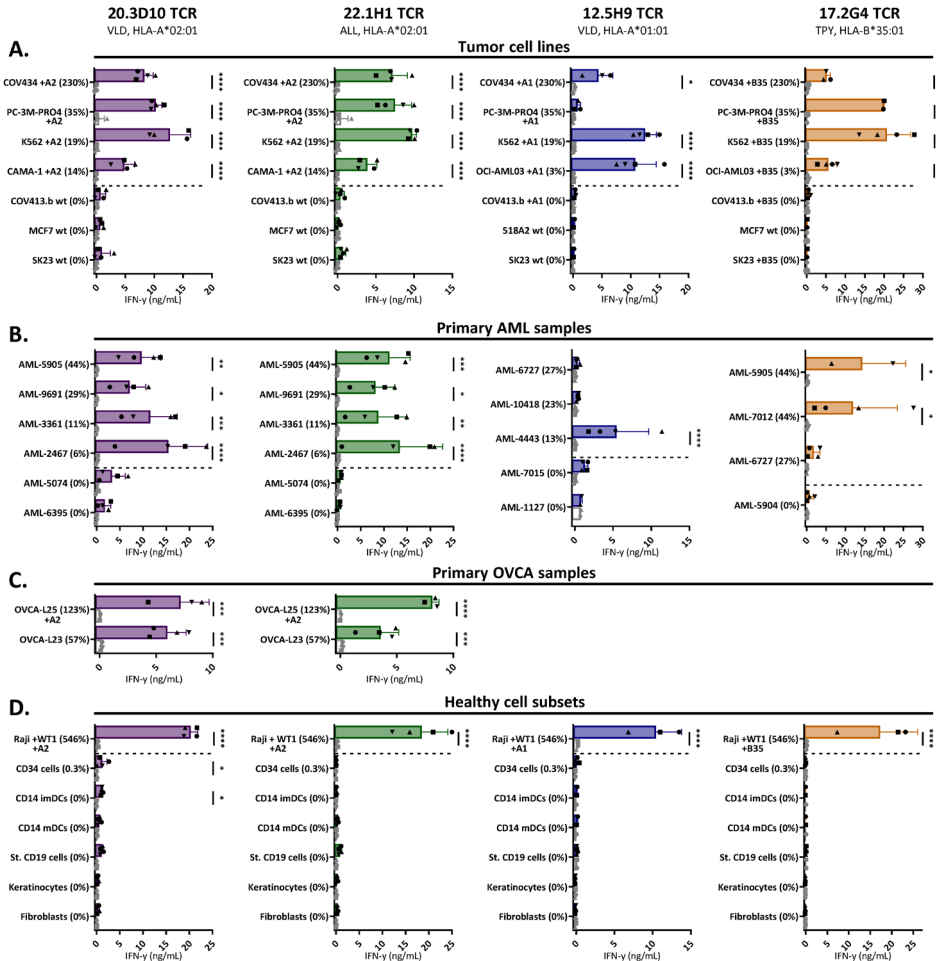


Figure 4. WT1-specific TCR-T cells recognize tumor cell lines and primary tumor samples, without recognition of healthy cell subsets

IFN- γ production (ng/mL) of purified TCR-T cells (CD8+) from four different donors cocultured overnight with (A) tumor cell lines (E:T = 1:6), (B) primary AML samples (E:T = 1:16), (C) primary OVCA samples (E:T = 1:6), and (D) several healthy cell subsets (E:T = 1:4 for keratinocytes, fibroblasts and CD14+, 1:6 for CD19+ cells, and 1:12 for CD34+ cells). The different symbols represent the TCR-T cells from four different donors, transduced with either the WT1-TCRs (colored bars, black symbols) or CMV-TCR (white bars, grey symbols). OVCA-L23 was ascites material passage 0 (wildtype HLA-A*02:01 +) and OVCA-L25 was ascites material passage 3 transduced with HLA-A*02:01 (35%). The healthy cell subsets used in (D) were isolated either from an HLA-A*02:01+ donor (for VLD/A2 and ALL/A2 TCRs) or from an HLA-A*01:01+ and HLA-B*35:01+ donor (for VLD/A1 and TPY/B35 TCRs). Also the other targets express the HLA-allele that presents the targeted peptide, either wildtype (wt) or the HLA-allele was introduced by transduction (+A2, +A1, +B35). In all figures the percentage relative WT1 expression is depicted, as determined by qPCR. (A-D) Data represent averaged duplicate values from four different donors. Mean and SD are depicted, WT1-TCR and CMV-TCR T cells were compared using two-way ANOVA, followed by Bonferroni post hoc analysis. Only significant results are shown. AML: acute myeloid leukemia, ANOVA: analysis of variance, OVCA: primary ovarian carcinoma, TCR: T-cell receptor.

WT1-SPECIFIC TCR-T CELLS EFFICIENTLY KILL PRIMARY AML SAMPLES AND OVCA CELL LINES

Finally, to investigate clinical potential of the four selected TCR-T cells, cytotoxic capacity against primary AML and OVCA patient samples and OVCA cell lines was measured in a 6-hour ⁵¹chromium release assay (Figure 5A-C). Raji pulsed with WT1 peptide were included as control (Figure 5D). Both 20.3D10^{VLD/A2}, 22.1H1^{ALL/A2}, and 17.2G4^{TPY/B35} TCR-T cells efficiently killed WT1 positive primary AML samples as well as OVCA patient samples and OVCA cell lines. The 12.5H9^{VLD/A1} TCR exhibited limited killing of AML samples but efficient killing of WT1 positive OVCA cell lines. Killing of all targets was observed by the allo-HLA reactive T-cell clones (Figure S3G-I).

Overall, these results demonstrate that TCR-T 20.3D10^{VLD/A2}, 22.1H1^{ALL/A2}, and 17.2G4^{TPY/B35} results in efficient killing of primary AML and OVCA patient samples and OVCA cell lines. To conclude, these TCRs are promising candidates for TCR gene transfer strategies for the treatment of patients with AML and OVCA. Given the lack of sufficient killing of primary AML samples, TCR 12.5H9^{VLD/A1} is only a potential candidate for TCR gene transfer strategies for patients with OVCA.

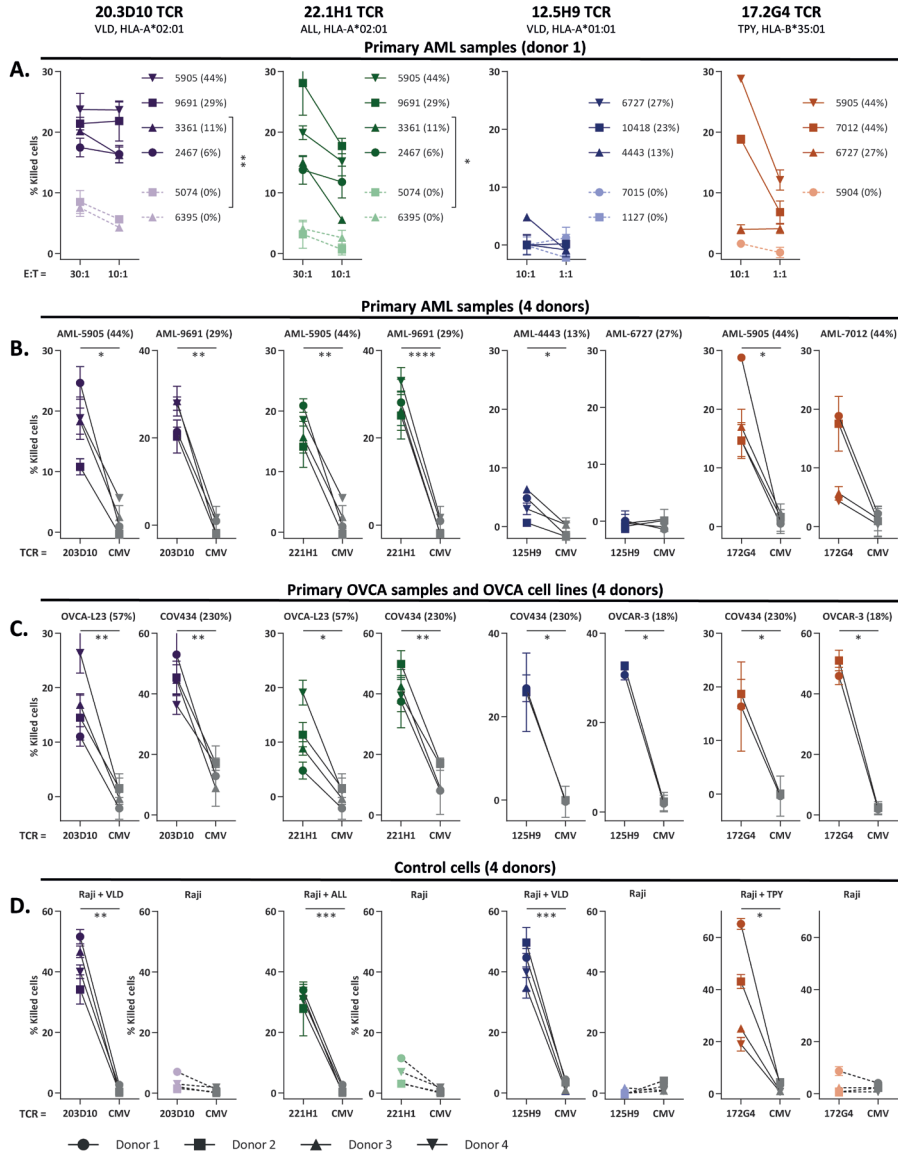


Figure 5. WT1-specific TCR-T cells efficiently kill primary AML and OVCA samples

Purified TCR-T cells (CD8+) were tested for cytotoxic capacity in a 6-hour ^{51}Cr -release assay against (A-B) primary AML patient samples, (C) primary OVCA samples and OVCA cell lines, and (D) Raji pulsed with WT1 peptide (0.2 μM). Percentage of killed cells is indicated on y-axis. (B-D) Cytotoxic capacity of the different WT1-TCR T cells was compared to CMV-TCR T cells. For VLD/A2, ALL/A2 TCRs killing capacity is depicted at E:T (effector-to-target) ratio of 30:1 and for VLD/A1 and TPY/B35 TCRs at E:T ratio of 10:1. All targets express the HLA-allele that presents the targeted peptide, either wildtype (wt) or the HLA-allele was introduced by transduction (+A2, +A1, +B35). Percentage relative WT1 expression is depicted, as determined by qPCR. (A) Mean and SD of technical triplicates are depicted for donor 1, WT1+ and WT1- AML samples and killing capacity of the WT1-TCR against these groups were compared using an unpaired t-test (two-sided). (B-D) Mean and SD of technical triplicates are depicted for four donors, WT1-TCR and CMV-TCR T cells were compared using a paired t-test (two-sided). Only significant results are shown. AML; acute myeloid leukemia, OVCA; primary ovarian carcinoma, TCR; T-cell receptor.

DISCUSSION

In this study, we describe eight WT1 peptides that were identified from the HLA class I associated ligandome of primary leukemia and OVCA patient samples. In a large-scale search for WT1-specific TCRs present in the allo-HLA repertoire, T-cell clones directed against five different WT1 peptides presented in three different HLA class I molecules were identified. No WT1-specific T-cell clones reactive against tumor cells could be identified that recognized peptides previously identified based on HLA peptide binding algorithms. By gene transfer of four of the five WT1-TCRs into CD8+ T cells, we analyzed the antitumor potential as well as the safety profile of the TCR-T cells, and our results demonstrated that these WT1-TCRs are promising candidates for TCR gene transfer strategies in patients with WT1-expressing tumors, including AML and/or ovarian carcinoma.

The HLA class I restriction molecules for TCR 20.3D10^{VLD/A2}, TCR 22.1H1^{ALL/A2}, TCR 12.5H9^{VLD/A1}, and TCR 17.2G4^{TPV/B35} are common. Of the global population 39% expresses HLA-A*02:01, 17% expresses HLA-A*01:01 and 8.5% expresses HLA-B*35:01.⁴¹ WT1-TCR-T cells induced potent killing of WT1 positive primary AML and OVCA patient samples and/or OVCA cell lines, without reactivity against healthy cell subsets and WT1 negative tumor cells. These results indicate that WT1-TCR-T cells do not exhibit off-target or on-target off-tumor toxicity. TCR 20.3D10^{VLD/A2} and TCR 22.1H1^{ALL/A2} demonstrated some cross-reactivity against HLA-A*33:01 and HLA-A*02:05, respectively. The frequencies of these HLA-alleles are however below 2%, implicating that only a small group of patients will not be suitable candidates for this particular TCR-based therapy.⁴¹

Our results demonstrate the relevance of establishing the HLA class I associated ligandome of tumors, since T-cell clones reactive against naturally WT1-expressing tumor cells were only isolated for peptides that were identified in the HLA class I associated ligandome. The most commonly used WT1 peptide is the RMF peptide presented in HLA-A*02:01. To our knowledge this peptide has never been found in peptide-elution databases of tumor samples⁴² and since we also failed to elute this peptide in our large set of WT1-expressing HLA-A*02:01 tumor samples, we question whether this peptide is efficiently processed and presented in WT1 positive tumors. Although high numbers of T-cell clones recognizing the RMF/A2 peptide were identified, recognition of naturally WT1-expressing tumor cell lines and primary AML patient samples was absent or very low. In contrast, VLD/A2 T cells were highly reactive against all WT1 positive tumor cell lines and primary AML patient samples (Figure 3). Only WT1 transduced Raji cells (546% relative WT1 expression) were highly recognized by the RMF/A2 T cells, demonstrating that the RMF/A2 peptide is processed and presented if WT1 is artificially overexpressed. These data indicate that limited antitumor reactivity can be expected of RMF/A2-specific T cells and this corresponds to observations of Jaigirdar et al that also suggested that the RMF/A2 peptide is not a suitable target for

T-cell based immunotherapies.²⁴ They demonstrated that a high-affinity RMF/A2-specific TCR, reactive against peptide loaded and WT1-transfected target cells, was not reactive against naturally WT1-expressing tumor cells due to absence of immunoproteasomes, resulting in no processing and presentation of the RMF/A2 peptide.²⁴ Moreover, RMF/A2-specific T cells were easily found in the autologous-HLA (auto-HLA) T-cell repertoire of most healthy individuals, but also no antitumor reactivity was observed.⁴³ Finally, our data also corresponds to the limited antitumor effects found in (pre)clinical studies targeting the RMF/A2 peptide.^{11,44} Overall, our data suggest that better clinical results may be achieved with the WT1 peptides that were identified in the HLA ligandome and for which potent T-cell clones were found.

The variation in antitumor reactivity against primary AML patient samples by the 12.5H9^{VLD/A1} TCR-T cells suggest variation in processing and presentation of HLA class I peptides between different samples, hypothetically due to differences in immunoproteasome expression. The 12.5H9^{VLD/A1} TCR-T cells efficiently recognize primary patient sample AML-4443, from which initially the VLD/A1 peptide was eluted (Table 1), suggesting optimal processing and presentation of this peptide in sample AML-4443. The lack of recognition of the other two AML samples, with higher WT1 expression, could be an indication of limited processing and presentation of this 12 amino acids long VLD/A1 peptide. It could also be an indication of alternative splicing or somatic mutations in the WT1 gene, which occur in 6-15% of de novo AML.^{45,46} Furthermore, also for the HLA-A*02:01 and HLA-B*35:01 positive primary AML patient samples a variety in antitumor reactivity was observed which could not be explained by WT1 expression. Both the ALL/A2 and TPY/B35 TCR-T cells and T-cell clones were reactive against all WT1 positive primary AML patient samples, whereas the VLD/A2 and HAA/B35 TCR-T cells and T-cell clones were not reactive against two samples (Figure 2). Our data therefore suggest the ALL/A2 and TPY/B35 peptides are processed and presented in more AML samples and therefore might be preferred targets to treat more AML patients. Nevertheless, more patient samples are needed to support this theory.

In this paper we demonstrate that potent and specific WT1-reactive T cells can be identified from the allo-HLA repertoire. Especially since these T cells were not subjected to the negative selection, we carefully evaluated the safety of our final TCRs. No on- and off-target toxicity was observed. Still, to eliminate unexpected serious adverse events of the WT1-TCR therapy, an additional clinical safety approach may be required. By co-transducing the WT1-TCR engineered T cells with a suicide switch, such as the inducible caspase-9 gene, prompt elimination of the engineered T cells can be induced. This approach was demonstrated for high-affinity PRAME-TCR transduced T cells *in vivo*.⁴⁷

The identified WT1-TCRs demonstrated potent antitumor reactivity against AML and OVCA tumors. By qPCR we confirmed WT1 expression in primary OVCA, AML as well as ALL patient

samples. Notably, not all AML patients are suitable candidates for WT1-TCR therapy, since only 60% of the AML patient samples expressed WT1. WT1 is additionally expressed in a broad variety of other tumors⁴⁸, indicating that also other solid tumors can be treated with WT1-TCR therapy. Besides the broad expression in various different tumors, several characteristics make WT1 an interesting target. WT1 promotes cancer progression through the induction of tumor angiogenesis and metastasis formation.⁷ In addition, WT1 is a strong predictor of leukemia relapse and is used as marker for minimal residual disease.⁴⁹ Also in MDS patients, overexpression of WT1 is associated with a higher risk for disease progression and AML transformation.⁵⁰ Finally, in solid tumors WT1 expression is also associated with poor prognosis, this is among others related to increased epithelial-to-mesenchymal transition.⁵¹

In summary, we identified the 20.3D10^{VLD/A2}, 22.1H1^{ALL/A2}, 12.5H9^{VLD/A1}, and 17.2G4^{TPY/B35} TCRs in a large-scale search for potent and specific WT1 TCRs present in the allo-HLA repertoire. We expect these TCRs to be a more potent option than the currently used WT1-TCRs from the auto-HLA repertoire. Also the naturally expressed WT1 peptides identified from the HLA class I associated ligandome of primary leukemia and OVCA patients, are expected to improve future vaccine and TCR gene therapy studies for WT1-expressing tumors.

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SUPPLEMENTAL TABLES AND FIGURES

Table S1. Overview of the materials included in HLA ligandome analyses

Nr	WT1 peptides eluted		Materials included in the HLA ligandome analyses							
	Peptide	HLA	BMI score	Name	WT1 expr.	Material	Amount	HLA-A	HLA-B	HLA-C
1	WTEGGSNHTGY	A*01:01	43	OVCA-G1	162%	Solid tumor	8 gram	A*01:01 A*11:01	B*08:01 B*35:01	C*04:01 C*07:01
			33	ALL-1833	9%*	PB	62x10 ⁹	A*01 A*03	B*18 B*35	C*04 C*07
			41	AML-4443	13%	PB	110x10 ⁹	A*01:01	B*08:01	C*07:01
			42	AML-10197	27%	PB	550x10 ⁹	A*01:01 A*02:01	B*08:01 B*44:03	C*07:01 C*16:01
2	VLDFAPPGASAY	A*01:01	36	AML-4443	13%	PB	110x10 ⁹	A*01:01	B*08:01	C*07:01
3	ALLPAVPSL	A*02:01	31	OVCA-L23	57%	Ascites	7x10 ⁹	A*02:01 A*26:01	B*38:01 B*44:02	C*05:01 C*12:03
4	VLDFAPPGA	A*02:01	26	OVCA-L23	57%	Ascites	7x10 ⁹	A*02:01 A*26:01	B*38:01 B*44:02	C*05:01 C*12:03
5	FGPPPSQA	A*02:01	42	OVCA-L23	57%	Ascites	7x10 ⁹	A*02:01 A*26:01	B*38:01 B*44:02	C*05:01 C*12:03
6	AQFPNHSFK	A*03:01	20	AML-10197	27%	PB	550x10 ⁹	A*01:01 A*02:01	B*08:01 B*44:03	C*07:01 C*16:01
7	HAAQFPNHSF	B*35:01	36	Cell line- COV362.4 HCL-4512	9% n.d.	Cell line Spleen	2x10 ⁹ 100x10 ⁹	A*03:01 A*02 A*29	B*40:01 B*35	C*03:04 C*04
8	TPYSSDNLY	B*35:01	41	ALL-2184	9%	PB	610x10 ⁹	A*11:01	B*35:01 B*40:02	C*02:02 C*04:01

Overview of the characteristics of materials included in the HLA ligandome analyses from which the WT1 peptides were identified. The materials are indicated per WT1 peptide, including their predicted HLA binding molecule based on NetMHC and best Mascot ion score (BMI). WT1 expression was determined by qPCR (*ALL-1833 by Illumina HT-12.0 microarray). OVCA: primary ovarian carcinoma, ALL: acute lymphoblastic leukemia, AML: acute myeloid leukemia, HCL: hairy cell leukemia, n.d. = not determined.

Table S2. Overview of the number of T-cell clones screened per healthy donor and T-cell clones reactive against WT1 peptide and/or transduced WT1

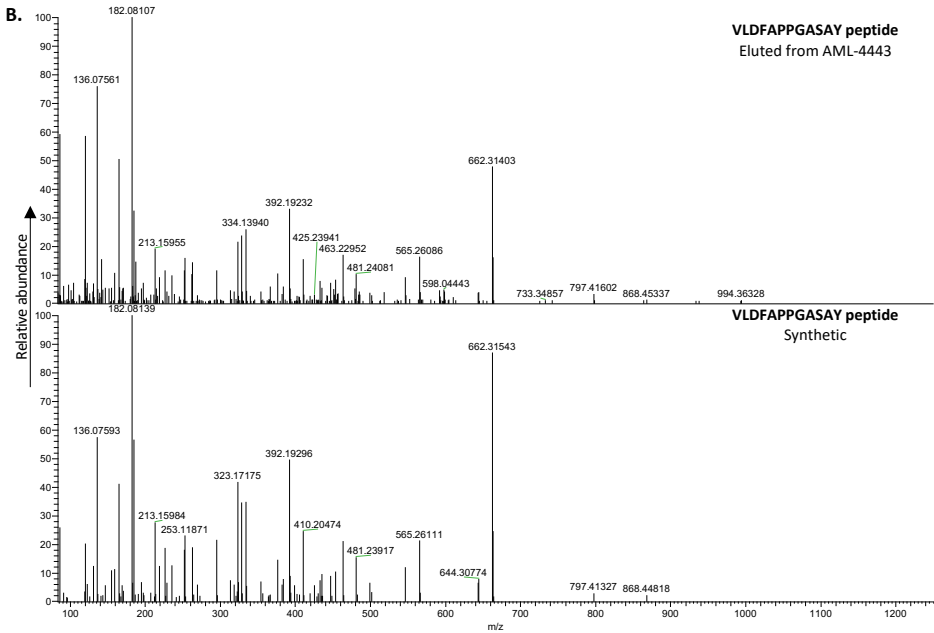
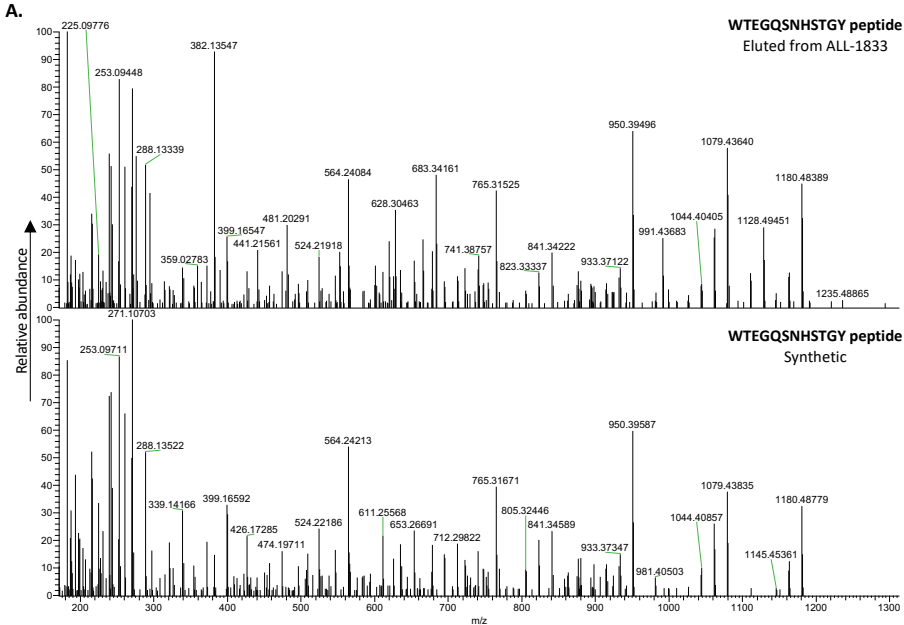
Name	HLA class I type	Number of PBMCs (x10 ⁶)	Screened T-cell clones	T-cell clones reactive against WT1 peptide	T-cell clones reactive against transduced WT1
OLA.01	A03, A29, B35, C unknown	595	69	13	2
OLA.02	A01, A31, B51, C04, C07	350	48	12	1
OLA.03	A01, A03, B13, B35, C04, C06	1150	102	18	0
OLA.04	A24, A29, B15, C03, C16	565	134	9	0
OLA.05	A03, B51, C03, C07	995	137	24	1
OLA.06	A24, B07, B27, C unknown	900	576	21	6
OLA.07	A03, A31, B07, B60, C07, C10	445	238	9	1
OLA.08	A24, A29, B44, B51, C unknown	795	658	38	8
OLA.09	A03, A32, B07, B44, C05, C07	825	145	11	1
OLA.10	A09, A28, B08, B18, C05, C07	755	520	20	0
OLA.11	A23, A31, B44, B51, C04, C15	490	315	13	0
OLA.12	A31, A28, B51, B60, C03	545	493	22	4
OLA.13	A24, A32, B55, B61, C02, C03	305	199	8	0
OLA.14	A24, A26, B39, B44, C02, C12	850	456	6	2
OLA.15	A24, A33, B14, B40, C03, C08	490	20	1	0
OLA.16	A30, A31, B18, B39, C05, C12	730	384	17	7
OLA.17	A32, A33, B14, B40, C03, C08	455	331	7	3
OLA.18	A01, A03, B57, B35, C04	575	87	24	3
OLA.19	A03, A24, B44, B63, C05	585	514	4	0
OLA.20	A03, A31, B50, B60, C06, C10	544	576	19	1
OLA.21	A01, A68, B51, B08, C07	724	269	11	0
OLA.22	A03, A24, B07, B35, C04, C07	710	267	11	2
OLA.23	A03, B07, B35, C04, C07	580	179	44	11
OLA.24	A32, B44, B18, C05, C07	565	316	14	2
OLA.25	A01, A25, B37, B51, C unknown	556	308	25	7
OLA.26	A01, A03, B07, B44, C07	310	233	29	7
OLA.27	A01, B39, B44, C05, C12	940	174	11	1
OLA.28	A01, A11, B15, B35, C unknown	800	168	20	1
		18,129	7,916	461	<u>71</u>

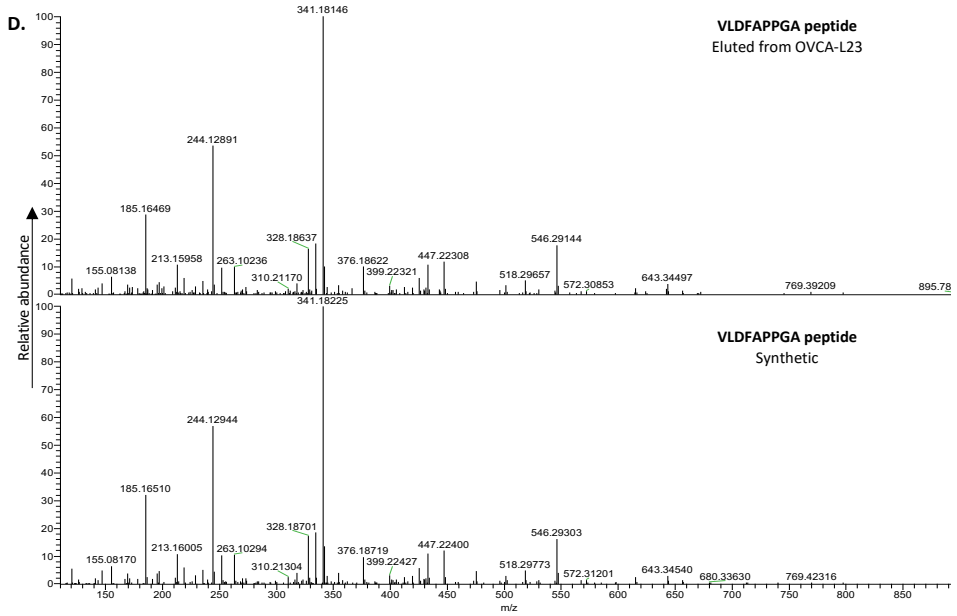
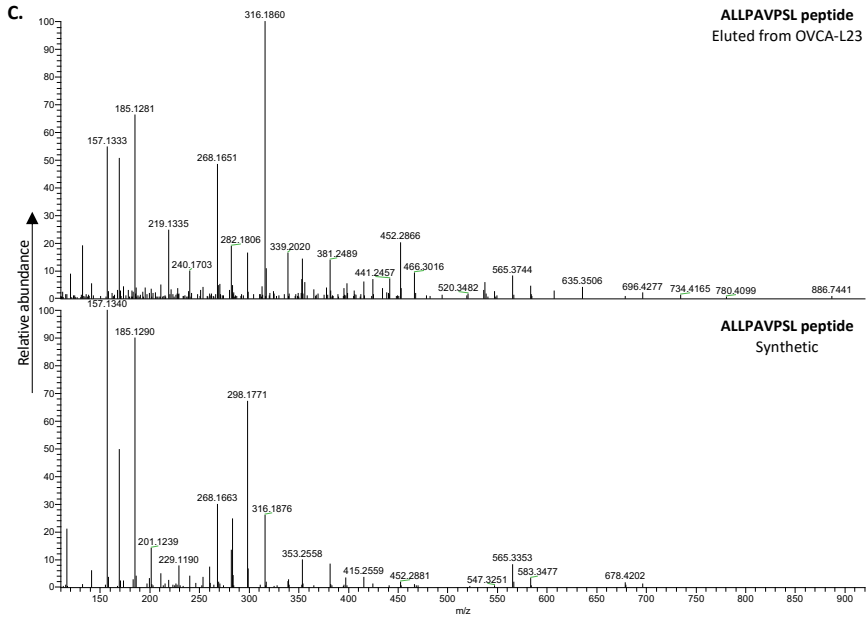
For each healthy donor, HLA class I typing, number of PBMCs, and expanded single-cell sorted T-cell clones are listed. In total 461 of 7916 screened T-cell clones were reactive against Raji pulsed with the WT1 peptide pool (1 μ M), and 71 clones were also reactive against Raji transduced with WT1. The reactivity is based on IFN- γ production (ng/mL) after an overnight coculture stimulation assay.

Table S3. Overview of T-cell clones reactive against transduced WT1 and those TCRs further analyzed

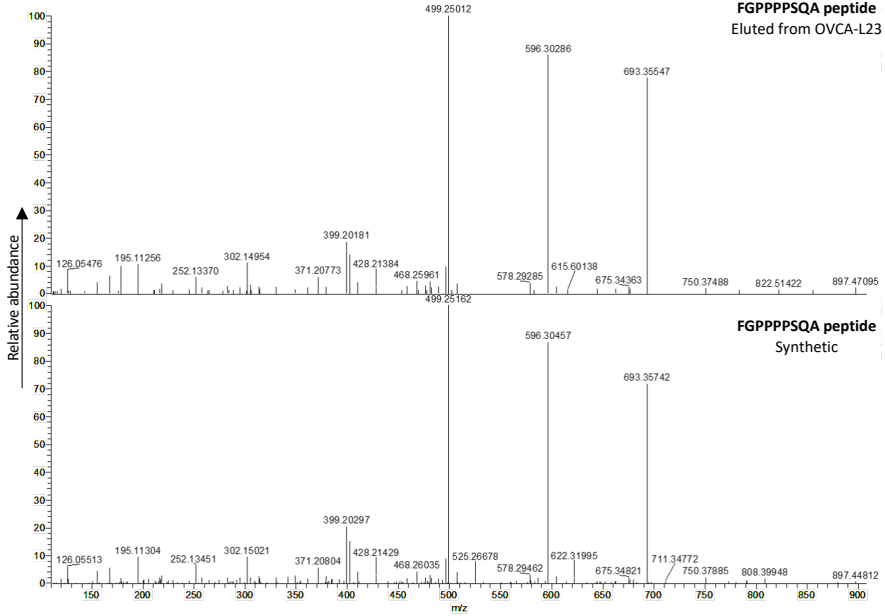
Nr	Peptide	HLA	Donors used for T-cell isolation	WT1-reactive T-cell clones	TCR analyzed
1	WTEGQSNHSTGY	A*01:01	23		
2	VLDFAPPGASAY	A*01:01	23	4	12.5H9
3	ALLPAVPSL	A*02:01	11	5	22.1H1
4	VLDFAPPGA	A*02:01	11	19	20.3D10
5	FGPPPPSQA	A*02:01	11	7	
6	AQFPNHSFK	A*03:01	5	1	
7	HAAQFPNHSF	B*35:01	19	18	
8	TPYSSDNLY	B*35:01	19	3	17.2G4
9	RMFPNAPYL	A*02:01	17	12	
10	CMTWNQMNL	A*02:01	17	2	
11	RWPSCQKKF	A*24:02	11		
12	CMTWNQMNL	A*24:02	11		
				71	4

Number of healthy donors included in the T-cell search is listed. In total 71 of the isolated T-cell clones were reactive against both Raji pulsed with the WT1 peptide pool and Raji transduced with WT1. The TCRs of four T-cell clones were sequenced and further analyzed.

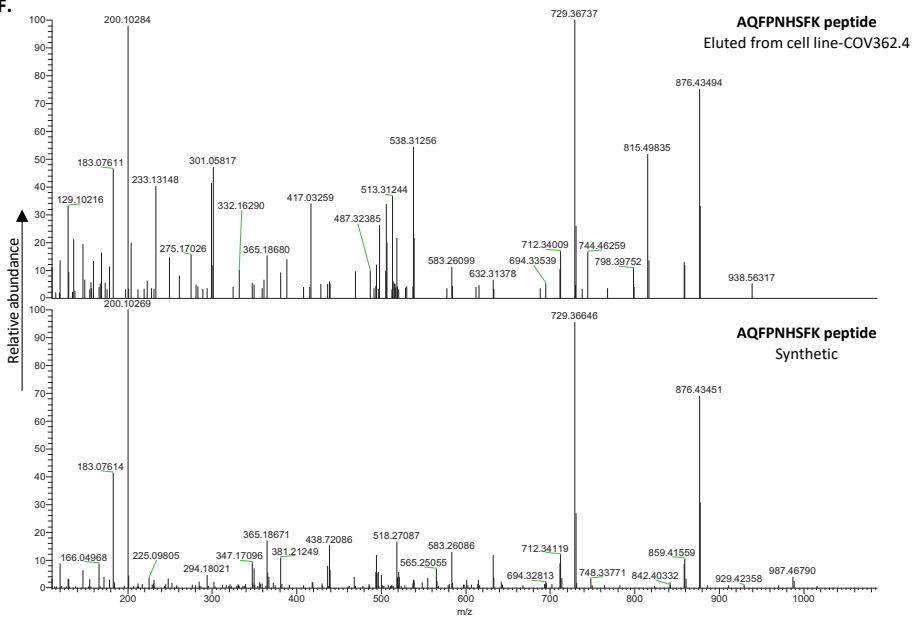




E.



F.



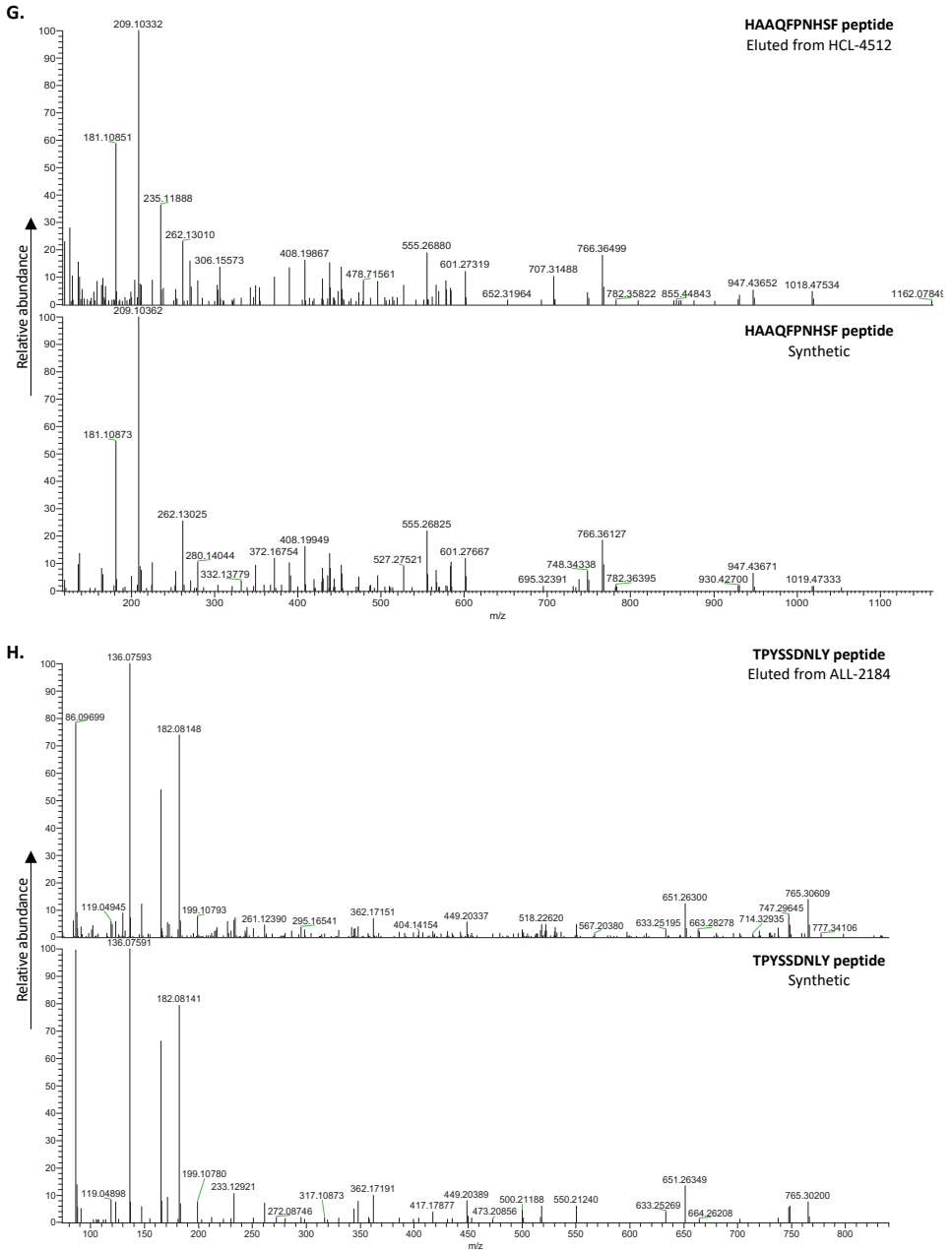


Figure S1. MS data validation of the eight WT1-derived peptides

The eight WT1 peptides identified in our HLA ligandome analyses were validated by comparing tandem mass spectra of eluted peptides and synthetic peptides. (**A – H**) For each peptide the tandem mass spectra of the eluted and synthetic peptides are shown, including the source of the eluted peptide.

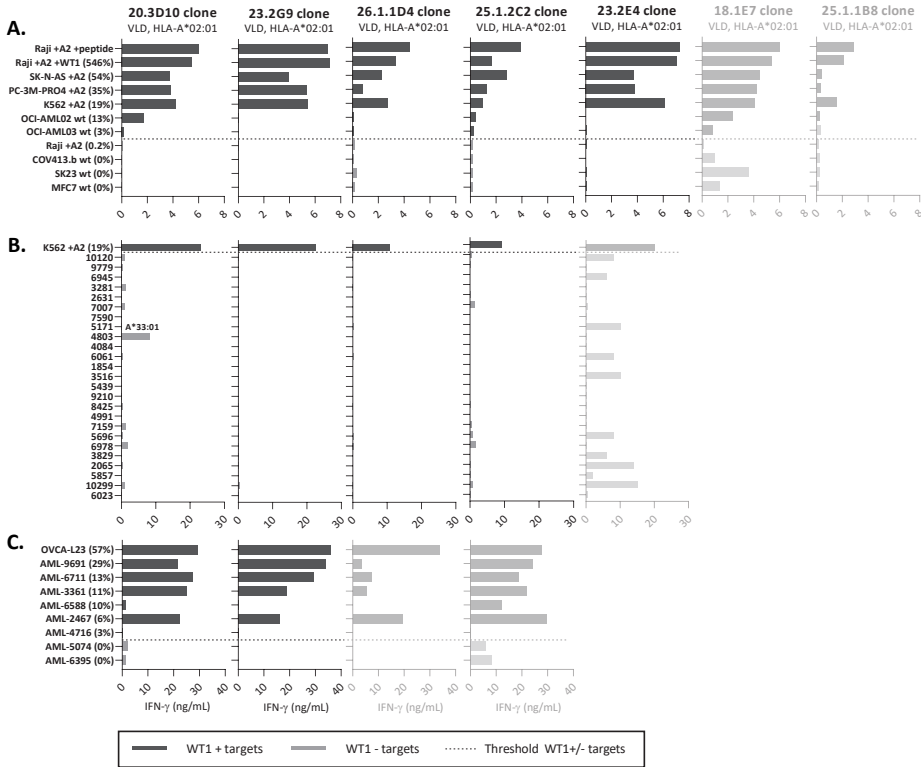


Figure S2. Screening approach to select specific and potent WT1-specific T-cell clones

Recognition patterns by 7 of the 19 T-cell clones recognizing the VLDFAAPPGA peptide presented in HLA-A*02:01. Clone 20.3D10 and 23.2G9 were selected as the most specific and potent T-cell clones. Excluded T-cell clones based on recognition in a panel are blurred. Recognition is based on IFN- γ production (ng/mL) after overnight coculture assays. **(A)** Panel with WT1+ and WT- tumor cell lines (E:T = 1:6). **(B)** Panel with 25 EBV-LCLs, expressing all frequent HLA alleles (with an allele frequency > 1%) present in the Caucasian population (E:T = 1:6). The HLA-allele is depicted if only one HLA-allele is recognized by the T-cell clone, meeting the requirement that all EBV-LCLs with this HLA-allele are recognized. **(C)** Panel with primary AML samples (E:T = 1:16) and OVCA patient samples (E:T = 1:6). All cell lines in panel A and C were HLA-A*02:01 positive, either wildtype (wt) or the HLA-allele was introduced by transduction (+A2). Percentage relative WT1 expression is depicted, as determined by qPCR. Dark grey bars depict high WT1+ targets and light grey bars the WT1- targets. Bars represent averaged duplicate values and are representative of two independent experiments.

Figure S3. Reactivities by allo-HLA reactive T-cell clones in the included screening panels [Figure on next page]

Reactivities of the positive control T-cell clones that are reactive against a housekeeping gene presented in HLA-A*02:01, HLA-A*01:01 or HLA-B*35:01. **(A-B)** IFN- γ production (ng/mL) against the tumor cell line panel and primary AML panel included in T-cell clone screenings, depicted in Supplementary data 5 and Figure 2. **(C-F)** IFN- γ production against the tumor cell line panel, primary AML panel, primary OVCA panel, and healthy cell subsets panel included in TCR-T cell screenings, depicted in Figure 4. **(G-I)** Killing percentages of the primary AML panel, OVCA cell line panel, and control panel included in the TCR-T cell screenings, depicted in Figure 5. Values and error bars represent mean and SD of technical duplicates and are representative of two independent experiments.

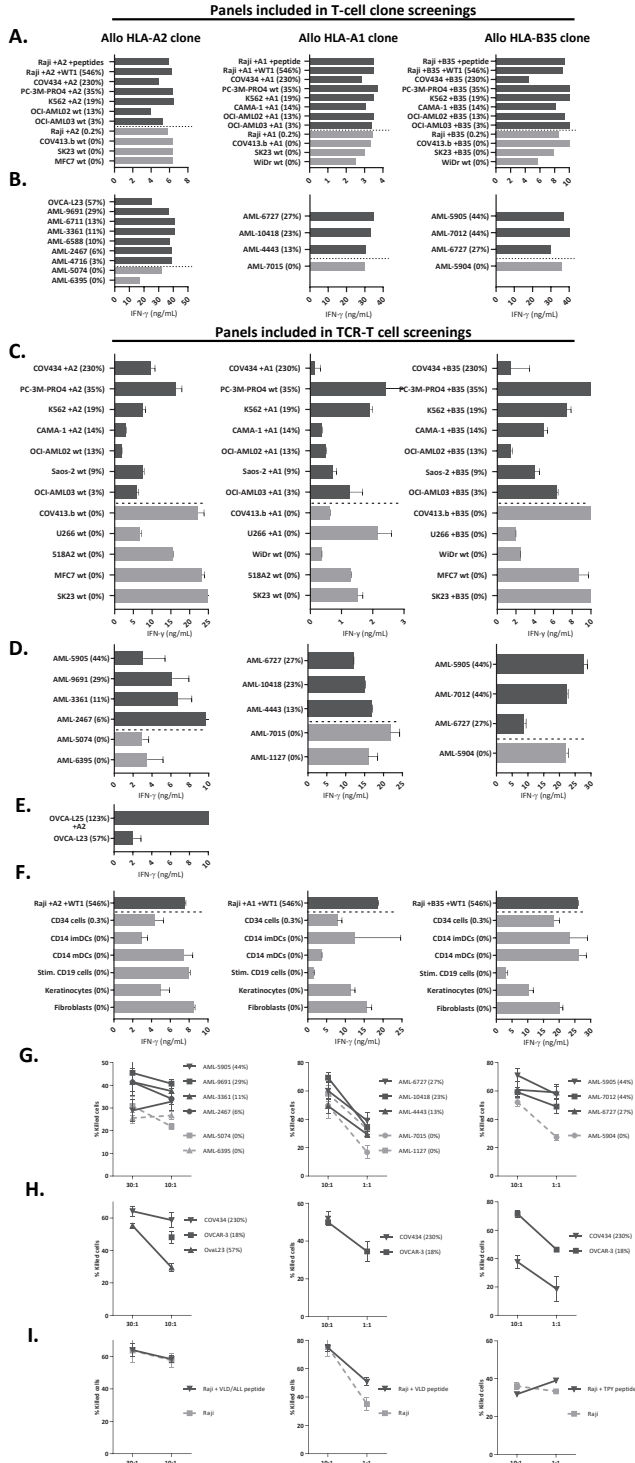


Figure S3. Reactivities by allo-HLA reactive T-cell clones in the included screening panels [Legend on previous page]

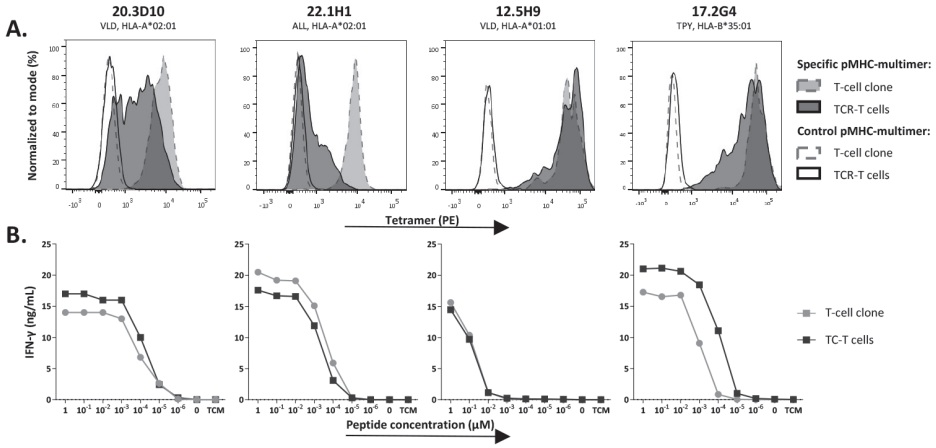


Figure S4. pMHC-multimer staining and peptide sensitivity of TCR-T cells and their parental T-cell clones
 T-cell receptors (TCRs) of the four most promising WT1-specific T-cell clones were constructed and introduced in CD8+ cells via retroviral transduction. Shown are representative results of two independent experiments and two CD8+ donors, at day 10 post isolation. **(A)** Flow cytometry plots of purified TCR-T cells and their parental T-cell clones stained with the specific and a control pMHC-multimer. **(B)** IFN- γ production (ng/ml) of T cells stimulated with Raji cells (transduced with HLA-A*01:01 or -B*35:01) or T2 cells (wildtype HLA-A*02:01 positive) pulsed with titrated peptide concentrations.

