Preclinical development of T-cell receptor-engineered T-cell therapy targeting the 5T4 tumor antigen on renal cell carcinoma

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Abstract
5T4 (trophoblast glycoprotein, TPBG) is a transmembrane tumor antigen expressed on more than 90% of primary renal cell carcinomas (RCC) and a wide range of human carcinomas but not on most somatic adult tissues. The favorable expression pattern has encouraged the development and clinical testing of 5T4-targeted antibody and vaccine therapies. 5T4 also represents a compelling and unexplored target for T-cell receptor (TCR)-engineered T-cell therapy. Our group has previously isolated high-avidity CD8+ T-cell clones specific for an HLA-A2-restricted 5T4 epitope (residues 17–25; 5T4p17). In this report, targeted single-cell RNA sequencing was performed on 5T4p17-specific T-cell clones to sequence the highly variable complementarity-determining region 3 (CDR3) of T-cell receptor α chain (TRA) and β chain (TRB) genes. Full-length TRA and TRB sequences were cloned into lentiviral vectors and transduced into CD8+ T-cells from healthy donors. Redirected effector T-cell function against 5T4p17 was measured by cytotoxicity and cytokine release assays. Seven unique TRA-TRB pairs were identified. All seven TCRs exhibited high expression on CD8+ T-cells with transduction efficiencies from 59 to 89%. TCR-transduced CD8+ T-cells demonstrated redirected cytotoxicity and cytokine release in response to 5T4+, on target-cells and killed 5T4+/HLA-A2+ kidney-, breast-, and colorectal-tumor cell lines as well as primary RCC tumor cells in vitro. TCR-transduced CD8+ T-cells also detected presentation of 5T4p17 in TAP1/2-deficient T2 target-cells. TCR-transduced T-cells redirected to recognize the 5T4p17 epitope from a broadly shared tumor antigen are of interest for future testing as a cellular immunotherapy strategy for HLA-A2+ subjects with 5T4+ tumors.

Keywords 5T4 · Trophoblast glycoprotein · Renal cell carcinoma · T-cell receptor · Adoptive cell therapy · Antigen processing

Abbreviations
5T4 Trophoblast glycoprotein, TPBG
ADCs Antibody–drug conjugates
CDR3 Complementarity-determining region 3
cPPT Central polypurine tract
CTLA4 Cytotoxic T-lymphocyte associated protein 4
IMGT International Immunogenetics Information System
MART-1 Melanoma antigen recognized by T-cells 1
MSCV Murine stem cell virus
mTOR Mammalian target of rapamycin
MVA Modified vaccinia virus Ankara
PTEC Proximal tubule endothelial cell
RCC Renal cell carcinoma
TAP Transporter associated with antigen processing
TET Tetramer
TKIs Tyrosine kinase inhibitors
TNF-α Tumor necrosis factor-α
TRA T-cell receptor α chain

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Introduction

Renal cell carcinoma (RCC) accounts for 90% of malignant neoplasms arising in the kidney in adults and is the eighth most common cancer in the United States, with an estimated 73,820 new cases in 2019 [1]. Despite the development of targeted molecular therapies, including tyrosine kinase inhibitors (TKIs) and mammalian target of rapamycin (mTOR) inhibitors, metastatic RCC is generally considered an incurable disease with a 5-year survival rate of only 12% [1]. However, metastatic RCC can be uniquely sensitive to systemic immunotherapy. Both high-dose interleukin-2 (IL-2) [2] and, more recently, combination immune checkpoint blockade with antibodies targeting programmed cell death-protein 1 (PD1) and cytotoxic T-lymphocyte associated protein 4 (CTLA4) [3] have been associated with complete radiographic responses in 5–9% of RCC patients. The anti-tumor effects produced by these agents are thought to be mediated by tumor-reactive T-cell responses.

While the antigens associated with immunotherapy mediated regression of RCC are not well defined, previous studies have identified 5T4 (trophoblast glycoprotein, TPBG) as an RCC-associated antigen of therapeutic interest. 5T4 is highly expressed by placental trophoblasts and a wide range of human carcinomas, including renal, prostate, pancreatic, ovarian, breast, cervical, gastric, and non-small cell lung cancer [4, 5]. Greater than 90% of RCC tumors over-express 5T4 and the expression is maintained on metastatic lesions [6]. 5T4 expression on tumors has been associated with a stem-cell phenotype subpopulation in human non-small cell lung cancers, nasopharyngeal carcinoma [7–9] and with epithelial–mesenchymal transition that may be related to cancer cell motility and metastatic spread [4]. 5T4 protein is undetectable or expressed at a very low level by healthy adult tissues [5, 6, 10].

The favorable expression pattern of 5T4 has encouraged the development and clinical testing of 5T4-targeted antibody–drug conjugates (ADCs) engineered with superantigen [11] or chemotherapy payloads [12] and a recombinant modified vaccinia virus Ankara (MVA) expressing the full-length 5T4 gene (MVA-5T4). MVA-5T4 is the most extensively studied 5T4-targeted therapy and has been applied to > 580 subjects with colorectal, prostate, and renal cancer [4]. Early phase clinical testing demonstrated MVA-5T4 was able to elicit 5T4-specific serological and T-cell responses in vaccinated cancer subjects [13]. 5T4-targeting by ADC or MVA-5T4 vaccine has not been associated with off-tumor on-target toxicities affecting healthy tissues. However, despite encouraging early phase data, none of these agents have gained regulatory approval as a cancer therapy.

Engineering T-cells to express foreign TCRs or chimeric antigen receptors (CARs) targeting tumor-associated antigens represents a therapy platform with the potential to massively expand tumor-reactive T-cells in cancer subjects. The recent clinical success of engineered T-cells expressing CARs specific for CD19 achieving complete remissions of refractory acute lymphocytic leukemia [14] and non-Hodgkin lymphoma [15] has created intense interest to extend engineered T-cells as a therapeutic modality to solid tumor targets. TCR-engineered T-cell therapy targeting the cancer/testis antigen NY-ESO-1 in melanoma and synovial sarcoma [16, 17], and more recently TCR engineered T-cells targeting human papillomavirus (HPV) antigens E6 or E7 in HPV+ cancers [18, 19] associated with partial tumor responses in some patients establish proof-of-concept for the therapeutic use of TCR engineered T-cells targeting a single tumor antigen to result in significant tumor regression.

5T4 represents a compelling and unexplored target for TCR-engineered T-cell therapy. Our group has previously isolated high-avidity CD8+ T-cell clones from both healthy and kidney cancer donors specific for an HLA-A2-restricted 5T4 epitope (residues 17–25; 5T4p17) [10]. In this study, we sequenced the CDR3s from the TRA and TRB genes isolated from these high-avidity 5T4p17-specific clones to identify unique TCRs recognizing 5T4p17. We have assessed 5T4p17-specific TCR-transduced T-cells from healthy donors for redirected recognition of 5T4p17 on target cells, including HLA-A2+ human tumor-cell lines and short-term in vitro cultures of primary RCC tumors expressing the 5T4 antigen.

Materials and methods

CDR3 domain sequencing for TRA and TRB genes from 5T4p17-specific CD8+ T-cell clones

Genomic DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) from 19 CD8+ T-cell clones specific for 5T4p17 presented by HLA-A2. High throughput-bulk sequencing of the T-cell receptor β chain was performed using the hsTCRB ImmunoSeq kit (Adaptive Biotechnologies, Seattle, WA) at survey level resolution [20] on the Illumina MiSeq platform (v3 150 cycle) in the Genomics Core Facility at the Fred Hutchinson Cancer Research Center. Repertoire analyses were conducted using the LymphoSeq R package (created by

| Term            | Definition                                                                 |
|-----------------|-----------------------------------------------------------------------------|
| TRA-CDR3        | CDR3 region from TCR-α gene                                                |
| TRAC            | Constant region of TCR-α gene                                               |
| TRB             | T-cell receptor β chain                                                     |
| TRB-CDR3        | CDR3 regions from the TCR-β gene                                            |
| TRBC            | Constant region of TCR-β gene                                               |
| WT1             | Wilms' tumor 1                                                              |
| WPRE            | Woodchuck posttranscriptional regulatory element                            |
Cloning full-length TRA and TRB sequences

Reference V- and C-gene open-reading-frames of TRA and TRB were obtained from the International Immunogenetics Information System (IMGT) [24, 25]. Codon optimized Vα and Vβ DNA fragments with corresponding CDR3 sequences were then synthesized by the GeneArt Strings DNA Fragments service (Invitrogen, Carlsbad, CA). Each DNA fragment included the following Gibson overhang sequences attached to both ends: Vα 5′: AGG AGACGTGGAGAAAAACCCCGTGTC; Vα 3′: ACA TCCAGAACCACCGACTGCTACCAGCTGGAG; Vβ 5′: TCCCCGAGCTCATAAAAGAGCCCACAACCCCTCCTGCGCCCGCGCCACCC; Vβ 3′: GTGTTCCCCCCAGAGTGGCCGGCTGTTGAG. The stop codon of constant region of TCR-β gene (TRBC) was deleted and the self-cleaving porcine teschovirus-1 2A sequence.

(P2A: GGTTCCGGAGCCACAGAACCCTCTCTCTCTG TTAAGGACGAGGAGGAGCGTGAGAAACCCCC GGTCCC) was incorporated between TRA and TRB, to ensure equal expression of α and β chains. Full-length TCRs were assembled using the Gibson Assembly® Ultra kit (SGI-DNA, La Jolla, CA). TCRs were then cloned into lentiviral vectors pRRLSIN, with the murine stem cell virus (MSCV) promoter to drive expression in human primary T-cells (pRRLSIN, pRSV-REV, pMD2-G, and pMDLg/pRRE were generous gifts of Dr. Philip Greenberg, Seattle, WA) [26]. Constructs were transformed into One Shot® TOP10 Chemically Competent E. coli (Invitrogen). Plasmid DNA was extracted using the Endotoxin-free Mini- and Midi-Prep DNA isolation kits (Qiagen).

Lentiviral packaging and T-cell transduction

Lenti-X 293T virus packaging cells (Clontech Laboratories, Mountain View, CA) were seeded at 60% confluence in RPMI-HEPES supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 1% penicillin/streptomycin (termed LCL medium). 5T4p17-specific TCR encoding lentivirus vectors were co-transfected with packaging plasmids (pRRLSIN-TRC: 1.5 μg, pRSV-REV: 1 μg, pMD2-G: 0.5 μg, and pMDLg/pRRE 1 μg) using the Effectene transfection reagent (Qiagen). Media was changed the next day; from day 2 post-transfection, and lentivirus containing supernatants were harvested each day for 2 days and passed through 0.45 μm filters. Viral supernatants were concentrated using Lenti-X viral concentrator (Clontech Laboratories), aliquoted and applied to T-cells for transduction or stored at −80 °C.

CD8+ T-cells were isolated from healthy donor PBMCs using a human CD8+ T-cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) and activated for 4 h with CD3/CD28 Dynabeads® human T-cell expander (Thermo Fisher Scientific, Waltham, MA). Viral supernatant was applied with 6 μg/mL polybrene (Sigma-Aldrich, St. Louis, MO) to T-cells and centrifuged at 1455 × g for 90 min at 35 °C. Transduction was repeated the following day. T-cell cultures were maintained in cytotoxic T-lymphocyte culturing media [27] with 50 U/mL recombinant human IL-2 (PeproTech, Rocky Hill, NJ) for 7 days.

Endogenous TRA knockout

Healthy donor CD8+ T-cells were stimulated for 2 days with CD3/CD28 Dynabeads® human T-cell expander (Thermo Fisher Scientific). crRNA–tracrRNA duplex was prepared the morning of electroporation. Constant region of TRA (TRAC) targeting crRNA (Alt-R® CRISPR-Cas9 crRNA: AGAGTCTCTCAGCTGGTACA [28], Integrated DNA technology, Inc., Coralville, IA) and tracrRNA (Alt-R® CRISPR-Cas9 tracrRNA, Integrated DNA technology, Inc.) were reconstituted to 200 μM with Nuclease-Free Duplex Buffer (Integrated DNA technology, Inc.) and mixed at equimolar concentrations. Oligos were then annealed by heating at 95 °C for 5 min in PCR thermocycler and slowly cooled to room temperature. Alternatively, control crRNA (Alt-R® CRISPR-Cas9 Control Kit, human, Integrated DNA technology, Inc.) was mixed with the tracrRNA the same manner. Duplexes and Cas9 Nuclease V3 (Integrated DNA technology, Inc.) were gently mixed and incubated at room temperature for at least 10 min. 1 million T-cells were resuspended in 20 μL primary-cell nucleofection solution (P3 Primary Cell Nucleofector™ Solution, Lonza, Basel, Switzerland). T-cells were added with Alt-R Cas9 Electroporation Enhancer (Integrated DNA technology, Inc.) to 4 μM, and
incubated with 5 μL duplex-Cas9 nuclease mix for 2 min. T-cells were electroporated using a 4D nucleofector (Lonza) with EH115 program. After nucleasection, T-cells were cultured at 10^6 cells per well in 200 μL prewarmed complete T-cell media. 6 h post nucleasection, T-cells were transduced with the corresponding lentivirus as described in the previous section.

Flow cytometry and T-cell expansion

To assess 5T4_p17-specific TCR expression, lentiviral-transduced T-cell cultures were stained by DAPI, 1:20 dilution of APC-Cy7-labeled anti-CD3 mAb, (clone RPA-T8; BD Biosciences, San Jose, CA), 1:20 dilution of FITC-labeled anti-CD8 mAb (clone RPA-T8; BD Biosciences) as well as 10 μg/mL of APC-labeled 5T4p17/HLA-A2 tetramer (TET, generated by the Immune Monitoring Core Laboratory at our center) and analyzed by flow cytometry (BD FACSymphony™, BD biosciences, San Jose, CA). Viable TET+CD8+CD3+ T-cells were flow-sorted (BD FACSAria™, BD Biosciences) for analysis by flow cytometry. T-cell and secondary PE-labeled anti-mouse IgG1 mAb (clone A85-1; BD Biosciences) were cultured in serum-free RPMI media supplemented with human β2-microglobulin (BosterBio) at 5 μg/mL and the test peptide. After overnight incubation at 37 °C, cells were stained with an APC-labeled HLA-A2-specific mAb (clone BB7.2, BD Biosciences) at dilution of 1:50 with DAPI and analyzed by flow cytometry.

Cytotoxicity and ELISA assays

For some experiments, T2- and LCL-targets were infected with wild-type MVA or MVA-5T4 (Oxford Biomedica, Oxford, UK) at a 10:1 multiplicity of infection in serum-free RPMI media at 37 °C for 1 h. Media were then adjusted to 10% serum. To confirm 5T4 expression on surface after MVA-5T4 infection, target cells were stained with a 5T4-specific mAb (clone 524744; R&D systems, Minneapolis, MN) at 1 μg/mL followed by a 1:50 dilution of a secondary PE-labeled anti-mouse IgG1 mAb (clone A85-1; BD Biosciences) for analysis by flow cytometry. T-cell and target-cell co-culture supernatants were harvested after 4 h to perform chromium-release assays as previously described [30]. Supernatants after 16 h of T-cell target-cell co-culture were assayed for interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α) by enzyme-linked immunosorbent assays (ELISA) according to the manufacturer’s protocol (BosterBio, Pleasanton, CA).

HLA-A2 stabilization assay

Synthetic peptide 5T4_p17 RLARLALVL, and alanine-substituted variant sequences ALARLALVL (R1A), RLAALALVL (R4A), RLAALALVL (L5A), RLAALALVL (L7A), RLARLALVL (V8A) at a purity > 90% (Genscript, Piscataway, NJ) were dissolved in 100% dimethyl sulfoxide (DMSO) and stored at 4 °C. Aliquots of 2 × 10^5 T2 cells were cultured in serum-free RPMI media supplemented with human β2-microglobulin (BosterBio) at 5 μg/mL and the test peptide. After overnight incubation at 37 °C, cells were stained with an APC-labeled HLA-A2-specific mAb (clone BB7.2, BD Biosciences) at dilution of 1:50 with DAPI and analyzed by flow cytometry.

Results

Sequencing TCRs from 5T4_p17-specific CD8⁺ T-cell clones reveals similarity in CDR3 region and V(D)J gene usage

Our group has previously characterized high-avidity 5T4_p17-specific CD8⁺ T-cell clones isolated from seven T-cell lines stimulated in vitro with varying concentrations of 5T4_p17. These lines were derived from leukaemapheresis products of four HLA-A2⁺ donors (three healthy donors, HD_A, HD_B, and HD_C; and one donor with 5T4⁺ metastatic kidney cancer, KCD_D, 10^7 responder T-cells for each condition) [10]. To assess the TCR-β gene usage, we sequenced CDR3 regions from the TCR-β gene (TRB-CDR3) expressed by 19 5T4_p17-specific CD8⁺ T-cell clones. Two clones had two TRB-CDR3 sequences—HD_A, clone #1 and HD_B, clone #20. In each case, one TRB-CDR3 sequence was identical to the other clones isolated from the same T-cell line (Table 1). Our hypothesis to account for two TRB-CDR3 sequences in these two “clones” was flow sorting of doublets representing a 5T4_p17-specific CD8⁺ T-cell that stained tetramer-positive adherent to an unrelated passenger T-cell. All three clones isolated from the HD_C/0.1 μg/mL-stimulated T-cells had a nonproductive TRB rearrangement (“null” allele) and a shared in-frame TRB-CDR3 sequence (CASSYMGPEAFF; Table 1).

Based on this interpretation, our analysis of 19 5T4_p17-specific CD8⁺ T-cell clones identified seven unique TRB-CDR3 sequences, with only one unique sequence corresponding to each of the seven originating T-cell lines. This result indicates the precursor frequency for each T-cell line with unique TRB-CDR3 could be as low as one cell out of the initial input 10^7 CD8⁺ T-cells. To further infer precursor frequency, we sequenced TRB-CDR3 from flow-sorted CD8⁺ T-cells purified from available donor leukaemapheresis products (HD_A, HD_B, and KCD_D) as well as two additional PBMC samples and a tumor-infiltrating lymphocyte sample also from donor KCD_D (Supplementary Table 1 and Supplementary Fig. 1). KCD_D was a 60-year-old male subject with metastatic clear-cell RCC, treated with high-dose IL-2 and multiple lines of targeted drug. Our analyses did not detect the corresponding TRB-CDR3 sequences associated with 5T4_p17-specificity from any of the samples.
collected from donors HD_A, HD_B, or KCD_D. These data suggest an upper boundary for precursor frequencies for 5T4p17-specific clones at < 1 in 1–1.5 × 10^5 CD8+ T-cells.

To identify the TCR-α gene CDR3 region (TRA-CDR3) pairing with each of the unique TRB-CDR3s, targeted single-cell RNA-Seq of the CDR3 regions of TRA and TRB genes was then performed. We discovered seven unique single TRA-CDR3 sequences that each paired with one of the seven TRB-CDR3s (Supplementary Table 2). Single-cell RNA-Seq analysis of HD_C, clone #17 confirmed both the nonproductive- and productive-rearrangement of TRB within each cell. Single-cell RNA-Seq analysis of HD_B, clone 20 resolved two separate cell populations, each expressing one of the identified TRB-CDR3 sequences consistent with our hypothesis of a flow-sorted doublet.

Our analysis did not reveal public TCR sequences shared between donors. However, we did observe CDR3 sequences with a high degree of homology. The TRA-CDR3 region of KCD_D-6 and HD_C-17 differed by only one amino acid at position 106 (serine of KCD_D-6, glycine of HD_C-17), while KCD_D-6 and HD_C-3 differed only at position 105 (alanine of KCD_D-6, serine of HD_C-3, Fig. 1a). V(D)J gene analysis with the IMGT V-quest [31, 32] also revealed common gene-segment usage among subsets of 5T4p17-specific TCRs. TRAV38-2 and TRAV145 were both shared by 4 of 7 TCRs, and TRBV6-3 and TRBJ2-7 were shared by 3 and 2 of 7 TCRs, respectively (Fig. 1c).

TCRs use a conserved mechanism to bind to HLA-A2 despite lack of consensus motifs in CDR1/CDR2 regions. Two unique positive-charged residues (R65 and K66) on the HLA-A2 α1-helix are crucial elements to interact with the negatively charged residues (Asp and Glu) in CDR1/CDR2 domains of HLA-A2-specific TCRs [33]. Therefore, V(D)J gene-segment usage for HLA-A2 specific tumor antigen recognition is biased [34]. Although the most frequently used V gene-segments in 5T4p17-specific clones (TRAV38 and TRBV6-3) are not among the most common HLA-A2 biased V genes, the CDR1 amino acid sequences of TRAV38 and TRBV6-3 are negatively charged at −2 and −0.9, respectively, consistent with preferential binding with

| Donor | [5T4p17] stimulation (µg/mL) | Clone | TRB-CDR3 sequence | Frequency (%) | TRA/B single-cell sequencing |
|-------|-----------------------------|-------|-------------------|--------------|----------------------------|
| HD_A  | 10                          | 1     | CASSELPA GGTEAFF  | 62.70        | −                          |
|       |                             |       | CASSELPA GGTEAFF  | 35.20        |                            |
|       |                             | 2     | CASSELPA GGTEAFF  | 97.94        | +                          |
|       | 0.1                         | 7     | CASSFFSNTGELFF    | 99.20        | −                          |
|       |                             | 8     | CASSFFSNTGELFF    | 98.17        | −                          |
|       |                             | 12    | CASSFFSNTGELFF    | 98.52        | −                          |
|       |                             | 15    | CASSFFSNTGELFF    | 97.94        | +                          |
| HD_B  | 10                          | 17    | CASQQVSGYEQYF     | 95.48        | −                          |
|       |                             | 19    | CASQQVSGYEQYF     | 99.54        | +                          |
|       | 0.1                         | 20    | CASSLTSQGFQPQHF    | 65.25        | +                          |
|       |                             |       | CASSPQGDNEAFF     | 34.06        |                            |
|       |                             | 21    | CASSPQGDNEAFF     | 99.02        | +                          |
|       |                             | 24    | CASSPQGDNEAFF     | 95.96        | −                          |
| HD_C  | 10                          | 1     | CASMDLA FKQYF     | 97.50        | −                          |
|       |                             | 3     | CASMDLA FKQYF     | 98.41        | +                          |
|       |                             | 6     | CASMDLA FKQYF     | 98.41        | −                          |
|       |                             | 11    | CASMDLA FKQYF     | 98.74        | −                          |
|       | 0.1                         | 17    | Null              | 63.66        | +                          |
|       |                             | 18    | Null              | 62.21        | −                          |
|       |                             | 20    | Null              | 62.80        | −                          |
| KCD_D | 10                          | 6     | CASFLITDTQYF      | 96.75        | +                          |

The bolded sequences were shared among clones from the same donor and peptide stimulation concentration.

TRB T-cell receptor beta, CDR3 complementarity-determining region 3, TRA T-cell receptor alpha, HD healthy donor, KCD kidney cancer donor.
HLA-A2 (Supplementary Table 2 and Fig. 1d). The amino acid sequences of 5T4p17-specific CDR3 regions are also negatively charged (Fig. 1d), except for CDR3α on HD_B-19 and CDR3β on HD_C-3, indicating a direct interaction of CDR3s and the positively charged 5T4p17 epitope (RLARLALVL, with two positively charged arginine residues).

5T4p17-specific TCRs exhibit robust and stable expression on CD8+ T-cells from healthy donors

We assembled the full sequences of TRA and TRB genes by adding reference V gene- and C gene-segments to the CDR3s (Supplementary Table 3; Supplementary Table 4). Codon optimized full-length TRA and TRB sequences were designed to incorporate cysteine point mutations in TRAC and TRBC, respectively (Supplementary Table 5), to crosslink lentiviral encoded 5T4p17-specific α and β chains and minimize mispairing with endogenous TCR counterparts (Fig. 2a) [35]. A P2A autocleavage site linked the full-length TRA and TRB genes with the orientation of TRB followed by TRA [36] to ensure equimolar production. CD8+ T-cells from peripheral blood of HLA-A2+ healthy donors were isolated and transduced with lentivirus encoding 5T4p17-specific TCRs. At day 7 post-transduction, 5T4p17-specific TCRs were detected by tetramer staining on 59–89% of transduced T-cells (Fig. 2b).

CD8+ T-cells expressing lentiviral-transduced TCRs exhibit redirected effector functions for 5T4p17/HLA-A2

No significant differences were observed in the transduction efficiency from three healthy donors (Supplementary Fig. 2). The mean fluorescent intensity of 5T4p17-specific TCR expression on flow-sorted tetramer+ cells following 12 days expansion was maintained at levels similar to the primary transduced cells (Fig. 2b). TCR transduced T-cells exhibited potent cytolytic activity for T2 targets pulsed with 10 nM 5T4p17 peptide without cross-reactivity for control HLA-A2 binding peptides (DDX3Y428–436 FLLDILGAT and UTY 148–156 KAFQDVLYV) (Fig. 2c) [37]. When tested for recognition of limiting dilutions of 5T4p17, six of the 7 TCR-transduced T-cell lines exhibited similar half-maximum lysis of 5T4p17 peptide-pulsed T2 targets at concentrations of 1–10 nM peptide (Fig. 2e). After overnight co-culture with T2 target-cells pulsed with 10 nM 5T4p17, TCR transduced CD8+ T-cells released robust amounts of TNF-α (Fig. 2d) again without cross-reactivity to control HLA-A2 binding peptides. The half-maximum release of TNF-α was observed with ~ 10 nM concentration of 5T4p17 peptide for six of the...
Fig. 2  5T4p17-specific TCRs expression on CD8+ T-cells from healthy donors and redirected effector functions against peptide-pulsed T2 targets. a Vector structure of 5T4p17-specific TCR assembly: TRB and TRA are transcribed under control of the MSCV promoter; P2A cleaves the primary transcripts to equal molar quantity of TRA and TRB in the cytosol. The enlarged graph indicates the gene segments and CDR3s, contributing to TRA and TRB sequence assembly. The relative location for the introduced transmembrane cysteines in TRAC and TRBC are indicated. b Cell surface expression of 5T4p17-specific TCRs stained by 5T4p17/HLA-A2 tetramer at day 7 post-transduction is shown in comparison to tetramer staining of the native T-cell clone expressing the corresponding TCR (upper panels). Cell surface expression of 5T4p17-specific TCRs on healthy donor T-cells is shown after sorting for 5T4p17/HLA-A2 tetramer+ cells and 12 days expansion (lower panels). CD8+ T-cells expressing 5T4p17-specific TCRs were tested for recognition of c T2 cells pulsed with 10 nM of 5T4p17 or control HLA-A2-binding peptides DDX3Y428–436 or UTY148–156 in a 4-h cytotoxicity assay. The effector:target ratio (E:T) was 10:1. d TNF-α release was measured by ELISA in culture supernatants harvested after 18 h co-culture of effector T-cells with T2 cells pulsed with 10 nM of 5T4p17 or the control peptide DDX3Y428–436 at 10:1 E:T. e Effector T-cells were tested for recognition of T2 cells pulsed with 5T4p17 peptide in a 4-h cytotoxicity assay at 10:1 E:T. f TNF-α release was measured by ELISA in culture supernatants harvested after 18 h co-culture of effector T-cells with T2 cells pulsed with 5T4p17 peptide at 10:1 E:T.
7 TCRs (Fig. 2f). Five of the seven TCR-transduced T-cell lines produced detectable TNF-α release above background at a 5T4p17 peptide concentration as low as 0.1 nM. Comparing with other TCRs, the HD_B-19 TCR-transduced T-cell lines required approximately tenfold higher peptide concentration for equivalent half-maximum lysis and TNF-α release. Similar observations were also made with IFN-γ release (Supplementary Fig. 3).

To closely examine the TCR-peptide/major histocompatibility complex (MHC) binding specificity for 5T4p17-specific TCRs, we synthesized five 5T4p17 peptide variants containing non-alanine residues substituted each to the nonpolar, aliphatic alanine residue, except for the two conserved anchor residues essential for HLA-A2 binding (RLARLALVL; lysine at position 2 and position 9) [38]. MHC binding affinities of alanine-substituted and proband 5T4p17 peptides were measured by cell surface HLA-A2 stabilization on T2 cells using flow cytometry (Fig. 3a). The 5T4p17, R1A, and R4A peptides stabilized HLA-A2 on the surface of T2 cells with a measurable increase of HLA-A2 staining. The alanine-substituted peptides L5A, L7A, and V8A showed no capacity to bind to HLA-A2 suggesting side chains at these positions may contribute to the peptide–MHC binding interaction.

We then performed a cytotoxicity assay with the seven 5T4p17-specific TCR expressing T-cell lines and T2 targets pulsed with the 5T4p17, R1A, or R4A peptides. Switching the positively charged arginine at position 4 to nonpolar alanine (R4A) disrupts T-cell recognition for this sequence by all 7 of the 5T4p17-specific TCRs (Fig. 3b). The R1A, the arginine to alanine changes at position 1 (R1A) differentially preserved 5T4p17-specific TCR recognition. Three of the seven 5T4p17 specific TCRs (HD_A-2, HD_A-15, and HD_B-21) killed T2 targets pulsed with R1A at high peptide concentration (Fig. 3b).

Fig. 3  Cytotoxicity of 5T4p17-specific TCR transduced CD8+ T-cells against T2 pulsed with alanine-substituted 5T4p17 peptides. a Binding of alanine-substituted 5T4p17 peptides to HLA-A2 was determined by stabilization of HLA-A2 on the surface of peptide-pulsed T2 cells. CMV pp65 is an HLA-A2+ T-cell epitope from CMV and serves as a positive control. Cell surface HLA-A2 was assessed by immunostaining and flow cytometry. b CD8+ T-cells expressing 5T4p17-specific TCRs were tested for recognition of T2 cells pulsed with the progenitor 5T4p17 peptide or HLA-A2 binding alanine-substituted variants in a 4-h cytotoxicity assay with a 10:1 E:T ratio.
**5T4<sub>p17</sub>-specific TCR transduced CD8<sup>+</sup> T-cells lyse 5T4<sup>+</sup>/HLA-A2<sup>+</sup> tumor targets**

We tested the cytotoxicity of 5T4<sub>p17</sub>-specific TCR-expressing T-cells against 5T4<sup>+</sup>/HLA-A2<sup>+</sup> RCC (A498, BB65, DOBSKI), breast cancer (MDA231) and a colon cancer-cell line (SW480) versus HLA-mismatched (SST548, BT20) or 5T4-negative target-cells (BB65-LCL). T-cells expressing each of the seven 5T4<sub>p17</sub>-specific TCRs demonstrated specific lysis above background levels with control targets for at least one 5T4<sup>+</sup>/HLA-A2<sup>+</sup> tumor line (Fig. 4a, b). Among the seven TCRs, T-cells expressing HD<sub>A-15</sub> and HD<sub>C-17</sub> consistently had the highest lytic potency against all 5T4<sup>+</sup>/HLA-A2<sup>+</sup> targets tested including RCC, breast cancer, and colon cancer-cell lines.

In some experiments, low-level lysis by 5T4 TCR-transduced T-cells was observed with HLA-mismatched (SST548, BT20) or 5T4-negative target-cells (BB65-LCL) versus control-target cell lysis seen with the originating T-cell clones. We speculated that low-level cytolytic activity for control-target cells could represent alloreactivity of the heterogeneous endogenous TCR repertoire expressed on the activated, CD8<sup>+</sup> effector population transduced with 5T4-specific TCRs. To test this hypothesis, we transiently introduced CRISPR/Cas9 guide-RNA targeting of endogenous TRAC by electroporation into healthy donor CD8<sup>+</sup> T-cells. CRISPR/Cas9 mediated TRAC targeting disrupted native TCR expression in 97.8% of electroporated T-cells indicated by loss of surface CD3 protein expression (Supplementary Fig. 4a, d). These CD3-negative T-cells were
then transduced with lentiviral vectors directing 5T4-specific TCR expression. Residual CRISPR/Cas9 activity was not anticipated to interfere with 5T4-TCR expression due to nucleotide changes in TRAC resulting from codon optimization of the 5T4-TCR sequences. The efficiency of lentiviral transduction and surface level of 5T4-specific TCRs remained unchanged compared with control electroporated T-cells (Supplementary Fig. 4b, c, e, f).

Transduced CD8⁺ T-cells with or without native TRAC disruption expressing HD_A-15 or HD_C-17 TCRs were re-tested for recognition of 5T4+/HLA-A2⁺ RCC (A498, BB65, DOBSKI) versus control targets demonstrating preserved lytic potency for the 5T4+/HLA-A2⁺ RCC lines and absent recognition of the control targets, including the HLA-mismatched SST548 RCC or 5T4-negative targets BB65-LCL and HLA-A2⁺/5T4⁻ fibroblast lines (Fig. 4c and Supplementary Fig. 5). The effector activity profile for 5T4-TCR transduced T-cells with native TRAC disruption was indistinguishable from the originating T-cell clones [10].

Transduced CD8⁺ T-cells with or without native TRAC disruption expressing HD_A-15 or HD_C-17 TCRs were then tested for recognition of primary 5T4+/HLA-A2⁺ RCC tumor cells versus autologous PTEC isolated from 4 donors (Supplementary Fig. 6). 5T4-TCR transduced T-cells with native TRAC disruption demonstrated specific tumor lysis for all 4 RCC tumors (5–30% specific lysis) with no lytic activity for the patient-matched syngeneic 5T4⁻ PTEC targets (Fig. 4d and Supplementary Fig. 6).

**5T4p17-specific TCR transduced CD8⁺ T-cells detect transporter associated with antigen processing (TAP)-independent processing of 5T4p17 in T2 cells**

One unique characteristic of 5T4p17 is its location within the signal sequence of 5T4 (Fig. 5a). Prior studies have shown that some T-cell epitopes within leader domains can load MHC-class I via a TAP-independent pathway as an alternative to the classic antigen presentation pathway mediated by proteasome degradation and TAP transport [39]. The T2 cell-line has bi-allelic deletions of chromosome 6 spanning the TAP1/2 genes [40]. Therefore, we tested the cytotoxicity of 5T4p17-specific TCR expressing T-cells against the T2 cell-line (HLA-A2⁺) versus a wild-type LCL target-line.

![Fig. 5 Cytotoxicity of 5T4p17-specific TCR-transduced CD8⁺ T-cells against MVA-5T4 infected targets.](image)

**Fig. 5** Cytotoxicity of 5T4p17-specific TCR-transduced CD8⁺ T-cells against MVA-5T4 infected targets. **a** Schematic of human 5T4 protein, p17–25 is in the signal sequence region. Trans: transmembrane region, Cyto: cytoplasmic region. **b** T2 cells and BB65-LCL were infected with MVA-WT or MVA-5T4, and cell surface expression of 5T4 was analyzed by immunostaining and flow cytometry 24 h after infection. The fraction of cells positive for cell surface 5T4 after MVA-5T4 infection is indicated. CD8⁺ T-cells expressing 5T4p17-specific TCRs were tested for recognition of **c** T2 and **d** BB65-LCL cells infected by MVA-WT, MVA-5T4, or uninfected cells pulsed with 10 nM 5T4p17 peptide in a 6-h cytotoxicity assay at a 10:1 E:T.
T2 targets (Fig. 5c), suggesting the presence of TAP1/2-
T2 cells above the background killing of MVA-WT infected-
T-cells consistently recognized and lysed MVA-5T4 infected
full-length 5T4 gene (MVA-5T4, Fig. 5b). 5T4p17-specific
infected with a recombinant vaccinia virus encoding the
TRA
analysis of the
as TRA-CDR3 amino acid sequences, identified a high
two donors (KCD_D-6, HD_C-3, and HD_C-17) utilized
donors for both
in patients and healthy donors specific for an HLA-A2 asso-
ciated Wilms' tumor 1 epitope  (WT1126–134) shared the usage
of physical constraints on TCR sequence to generate high
specificity, lytic potency, and off-target reactivity for the
t-cell therapy, we assessed the expression profile, antigen
TCR recognition of the 5T4p17 epitope sequence.
healthy  CD8+ T-cells, is readily detectable at the cell sur-
five TCRs. Each TCR efficiently assembles in transduced-
specificity, lytic potency, and off-target reactivity for the
these TCRs were distinguished when 5T4p17-specific TCR-
domed. The 8-mer 5T4p18–25 sequence is also unique to 5T4,
to RCC, breast, and colon cancer tumor lines and primary
of the TCR's also differed for recognition of an alanine-
specific TCRs for a heterologous protein containing a shared
An NCBI peptide blast search reveals that the 9-mer
5T4p17-specific TCR transduced CD8+ T-cells is
For example, CD8+ T-cells in patients and healthy donors specific for an HLA-A2 asso-
ated Wilms' tumor 1 epitope (WT1126–134) shared the usage of
TRBV3, 6, 7, 20, 27 [41]; and CD8+ T-cell clones specific
for the HLA-A2 associated Melanoma antigen recognized by T-cells 1 (MART-126–35) epitope used
for the HLA-A2 associated Melanoma antigen recognized by T-cells 1 (MART-126–35) epitope used
our original T-cell culture experiments.
Our sequence analyses have demonstrated that naturally occurring 5T4p17-specific T-cells are present at very
low-frequency. Our studies set an estimate of precursor

Discussion

In this report, we have identified 7 unique TCRs with spec-
ificity for the 5T4p17 epitope presented by HLA-A2. Our analysis of the TRA and TRB gene segment usage, as well
as TRA-CDR3 amino acid sequences, identified a high
degree of homology between TCRs. We identified clones with shared V-and J-gene segments isolated from unrelated
donors for both TRA and TRB. Three similar α-chains from
two donors (KCD_D-6, HD_C-3, and HD_C-17) utilized
the same TRAV38-2/TRAJ45 and TRBV6-3, differing by only
one amino acid within TRA-CDR3. It has been shown that TCRs against the same tumor-associated epitope use biased
sets of variable gene segments. For example, CD8+ T-cells
in healthy donors for both TRA and TRB.

TRAC T-cell clones. Using CRISPR/Cas9 mediated TRAC targeting and disruption of native TCR expression, we provide direct
evidence that allo-reactivity of the heterogeneous endogenous
TCR repertoire expressed on the activated, CD8+ effector
population transduced with 5T4-specific TCRs accounts
for 5T4 independent-target reactivity. 5T4-TCR transduced
T-cells with native TRAC disruption showed no lytic ac-
activity for 5T4-negative cells representing healthy tissues that
included LCL, fibroblast, and PTEC targets. TRAC disrup-
tion may also prevent the risk of TCR chain mispairing that
could result in unexpected auto-reactivity [43]. Whether dis-
ruption of native TCR expression in transduced T-cells could
augment specific antigen recognition of transduced TCRs by
eliminating competition for TCR signaling co-factors is an
area of ongoing investigation.

We searched a database of all publicly available
TRB-CDR3 sequences curated by our research group
currently  > 320 million unique TRB-CDR3s) for
5T4p17-associated TRB-CDR3 sequences. All seven of the
TRB-CDR3s sequences from 5T4p17 specific TCRs were
identified in non-RCC bearing individuals. In one public
dataset of TRB-CDR3 sequencing from 678 healthy individ-
uals [44], identical TRB-CDR3 sequences to HD_A-15 and
HD_C-17 were found in 44 (12 HLA-A2+) and 5 (1 HLA-
A2+) healthy bone marrow samples, respectively. The natu-
ral occurrence of these TRB-CDR3s in HLA-A2+ healthy
donors suggests the possibility for 5T4-reactive TCRs to
circulate in the repertoire of a subset of healthy donors in
addition to the individuals providing research samples for
our original T-cell culture experiments.

Our sequence analyses have demonstrated that natu-
ally occurring 5T4p17-specific T-cells are present at very
low-frequency. Our studies set an estimate of precursor

(BB65-LCL, HLA-A2+). Both T2 cells and BB65-LCLs
express comparable 5T4 protein on their surface when
infected with a recombinant vaccinia virus encoding the
full-length 5T4 gene (MVA-5T4, Fig. 5b). 5T4p17-specific
T-cells consistently recognized and lysed MVA-5T4 infected
T2 cells above the background killing of MVA-WT infected-
T2 targets (Fig. 5c), suggesting the presence of TAP1/2-
independent presentation of the 5T4p17 epitope in T2 cells.
The specific lysis for 5T4-expressing T2 targets was always
less than for 5T4-expressing BB65-LCL (Fig. 5d). These
results suggest that in the antigen processing-competent
target-cells, 5T4p17-processing and MHC-class I presenta-
in RCC tumor in short-term culture. T-cells transduced with
HLA-A15 or HD_C-17 TCRs demonstrated the highest
most consistent specific lysis for the tumor lines tested.

An NCBI peptide blast search reveals that the 9-mer
5T4p17–25 sequence is unique to 5T4 in the human pepti-
dome. The 8-mer 5T4p18–25 sequence is also unique to 5T4,
indicating a potential cross-reactivity of R1A 5T4p17-specific
TCRs for a heterologous protein containing a shared
5T4p18–25 sequence is not expected. In performing functional
testing of 5T4p17-specific TCR transduced CD8+ T-cells, we
noted low-level cytolytic activity for 5T4+ or HLA-A2+ tar-
get cells that had not been observed with the originating
T-cell clones. Using CRISPR/Cas9 mediated TRAC targeting
and disruption of native TCR expression, we provide direct
evidence that allo-reactivity of the heterogeneous endogenous
TCR repertoire expressed on the activated, CD8+ effector
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Our sequence analyses have demonstrated that natu-
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frequency for these T-cells as low as 1 in $10^7$ CD8+ T-cells in responding donors [10]. These data are consistent with immune monitoring studies performed with MVA-5T4 vaccination that have also revealed a heterogeneous response phenotype for induced ST4-specific T-cell immunity and low titer of measured T-cell responses [13]. Inadequate T-cell priming and expansion likely accounts for the failure of the tumor antigen-specific cancer vaccines MVA-5T4 and IMA901 to show improved survival for RCC patients in phase III clinical trials [45, 46].

In contrast to ST4-specific vaccination, engineered autologous T-cells expressing CARs or TCRs specific for ST4 represents an emerging therapy platform that, in principle, will facilitate a massive expansion of tumor-reactive T-cells in treated subjects. ST4 is a transmembrane antigen and can, therefore, be targeted by either CAR or TCR-based strategies. Preclinical assessment of ST4-CARs has demonstrated recognition of human nasopharyngeal carcinoma in vitro [8] and ovarian cancer in a murine xenograft model [47]. A potential advantage for TCR targeting of the ST4p17 epitope is its location within the signal sequence of the ST4 protein. It has been well established that a subset of T-cell epitopes within leader domain sequences can load class I MHC by a TAP-independent processing pathway [39]. Deficient TAP expression is a common tumor-associated phenotype thought to contribute to tumor escape from immune surveillance [48, 49]. We observed specific lysis of ST4p17-specific TCR-transduced CD8+ T-cells indicating the ST4p17 epitope can be processed by a TAP-independent pathway. The robust specific lysis of ST4p17-specific TCR-transduced CD8+ T-cells for ST4 infected LCL targets also suggests the intriguing clinical scenario that subjects treated with TCR-engineered ST4p17-specific T-cells could be subsequently vaccinated with MVA-5T4 to re-stimulate the transduced effector T-cells against the ST4 target in vivo.

In summary, TCR-engineered ST4p17-specific CD8+ T-cells are of interest for future testing as a cellular-immunotherapy product in subjects with ST4+ tumors.

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Author contributions SST, EHW, and YX designed the study; YX, AJM and MJC performed the experiments; AMHT performed the TCRB sequencing; YX and DGC performed the single-cell sequencing analysis; YX and SST wrote the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest A provisional patent application was filed by Yuexin Xu, Edus H. Warren and Scott S. Tykodi regarding TCRs described in this study. The other authors declare that they have no conflict of interest.

Ethical approval and ethical standard All study protocols and donor consent documents were approved by the Institutional Review Board of Fred Hutchinson Cancer Research Center, Seattle, Washington, USA and included protocols 1495 (skin biopsy), 999.209 (skin biopsy), 868 (healthy donor leukapheresis), 1246 (cancer patient leukapheresis), and 1810 (RCC tumor, normal kidney, peripheral blood).

Informed consent Written informed consent was obtained from all individual donors of biological samples for research use included in this study. Kidney cancer donor D consent for the collection of RCC tumor tissue for study 1810 also authorized collection of clinical data from the donor’s electronic medical record, including treatment history and disease status.

Cell line authentication The cell lines T2, A498, MDA-231, BT-20, and SW480 were obtained from American Type Culture Collection (Manassas, VA). The cell line lenti-X 293T virus packaging cells was obtained from Clontech Laboratories (Mountain View, CA). The RCC tumor lines LB1828, BB65, DOB5KI, and the BB65-LCL line were a generous gift from Dr. Benoît Van den Eynde (Ludwig Institute for Cancer Research, Brussels, Belgium). The TM-LCL line was a generous gift from Dr. Phillip D. Greenberg and Dr. Tom B. Schmitt (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). The phenotypes of these lines were verified by the source before arriving at our facility. The phenotypic characterization of RCC tumor line SST548 was previously verified [10]. Paired RCC tumor and PTEC cultures from four clear-cell RCC tumors and adjacent normal kidney cortex, as well as three fibroblast lines derived from skin biopsies of three additional donors were established following specific cell culture protocols to ensure propagation of the targeted cell types [50–52]. Expression of the ST4 tumor marker on the four primary RCC lines was verified by flow cytometry, as shown in Supplementary Fig. 6. No ST4 expression was detected on any fibroblast or PTEC lines [10]. PCR-based HLA-typing of genomic DNA was performed for all cell lines to select for HLA-A2 expression [10]. Mycoplasma was routinely tested. Phenotypes for cell morphology and growth rate were monitored regularly and did not change over time. All the experiments were performed with cells at a low passage number (≤ 10).

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