HHS Public Access

Author manuscript

Cancer Immunol Res. Author manuscript; available in PMC 2019 May 01.

Published in final edited form as:

Cancer Immunol Res. 2018 May ; 6(5): 594–604. doi:10.1158/2326-6066.CIR-17-0434.

Rapid construction of antitumor T-cell receptor vectors from frozen tumors for engineered T-cell therapy

Takemasa Tsuji[1,2], **Akira Yoneda**[1], **Junko Matsuzaki**[1,2], **Anthony Miliotto**[1], **Courtney Ryan**[1], **Richard C. Koya**[1,2], and **Kunle Odunsi**[1,2,3]

[1]Center for Immunotherapy, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

[2]Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

[3]Department of Gynecologic Oncology, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

Abstract

T cells genetically engineered with tumor antigen-specific T-cell receptor (TCR) genes have demonstrated therapeutic potential in patients with solid tumors. In order to achieve broader application, an efficient method to identify TCR genes for an array of tumor antigens and HLA restriction elements is required. Here, we have developed a method to construct a TCR-expression library from specimens, including frozen tumor biopsies, that contain antigen-specific T cells. TCR-expressing cassettes were constructed and cloned in a retroviral plasmid vector within 24 hours by unbiased PCR amplification of TCR α and β chain variable regions assembled with TCR constant regions. The method was validated by constructing TCR-expressing vectors from tumor antigen-specific T-cell clones and functionally assessing TCR gene-transduced T cells. We applied this method to frozen ovarian tumor specimens that were infiltrated by tumor antigen-specific T cells. The tumor-derived TCR libraries were expressed in peripheral T cells from healthy volunteers and screened for tumor antigen-specific TCR pairs with the use of an MHC/peptide tetramer reagent. Tumor antigen-specific TCR-expressing transgenes were recovered from isolated tetramer-positive T cells. Peripheral T cells that were engineered with library-derived TCR gene showed potent therapeutic antitumor effect in a tumor xenograft model. Our method can efficiently and rapidly provide tumor-specific TCR-expressing viral vectors for the manufacture of therapeutic and personalized antitumor T-cell products.

Corresponding Author: Kunle Odunsi, Center for Immunotherapy, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA. Phone: 1-716-845-8455; Fax: 1-716-845-1595; kunle.odunsi@roswellpark.org.

Disclosure of Potential Conflicts of Interest

T. Tsuji, R.C. Koya, and K. Odunsi are inventors of a patent application by RPCI regarding the TCR cloning. Other authors have no conflict of interest.

Author's Contribution

Conception and design: T. Tsuji, R.C. Koya, K. Odunsi
Development of methodology: T. Tsuji
Acquisition of data: T. Tsuji, A. Yoneda, J. Matsuzaki, A. Miliotto, C. Ryan, K. Odunsi
Analysis and interpretation of data: T. Tsuji, A. Yoneda, J. Matsuzaki, R.C. Koya, K. Odunsi
Writing and review of the manuscript: T. Tsuji, A. Yoneda, J. Matsuzaki, A. Miliotto, C. Ryan, R.C. Koya, K. Odunsi
Administrative, technical, or material support: J. Matsuzaki, A. Miliotto, C. Ryan, K. Odunsi
Study supervision: K. Odunsi

Introduction

Tumor antigen-specific T cells recognize cancer targets via heterodimeric T-cell receptors (TCR) that recognize tumor antigen-derived peptides loaded on major histocompatibility complex (MHC) molecules on cancer cells. Diverse sequences in both TCR α and β chains, especially in their complement-determining region 3 (CDR3), determine MHC restriction and peptide specificity. Adoptive transfer of autologous tumor antigen-specific T cells into cancer patients is a promising therapeutic strategy for treatment of cancer patients (1–7). Because it is difficult to expand sufficient numbers of autologous tumor antigen-specific T cells from patients, methods have been developed to engineer peripheral bulk T cells to express tumor antigen-specific TCR genes(8–10). It has been widely demonstrated that TCR gene-engineered T cells have antitumor effects comparable to the parental T-cell clones against cancer targets. Clinical trials testing TCR gene-engineered T cells have demonstrated feasibility, safety and therapeutic effects in multiple tumor types (11–14). However, only a limited number of therapeutic antitumor TCR genes have been developed, which limits the broad application of this therapeutic strategy to cancer patients (15, 16).

Traditionally, tumor antigen-specific TCR α and β chain genes are obtained from well-characterized tumor antigen-specific T-cell clones expanded *in vitro*. However, establishing tumor antigen-specific T-cell clones targeting a broad array of tumor antigens and MHC restriction elements is laborious and technically challenging in a high-throughput manner. Single-cell approaches such as single-cell PCR (17–21) and emulsion PCR (22, 23) can identify tumor antigen-specific TCR pairs. However, obtaining high-quality antitumor T cells from cancer specimens requires collection and processing of large amounts of freshly resected surgical specimens, which may not be feasible in all patients. Alternatively, next generation sequencing (NGS) has been utilized to identify paired TCR α and β chain sequences from frozen tumor specimens (24–27). In this method, sets of TCR α and β sequences for tumor-infiltrating T cells are obtained, and pairing of TCR α and β chain genes is predicted based on matched frequencies in each specimen. Estimating absolute frequencies for TCR genes is still challenging with this approach because many T cells express two TCR α chain genes (28). In both single cell– and NGS-based approaches, end-point results are often nucleotide sequences for many candidate TCR pairs. Laborious procedures such as synthesizing the TCR-expressing cassettes, cloning in expression vectors, and testing reactivity against target antigens are then required to identify candidate therapeutic TCR genes. More rapid identification of tumor-reactive TCR genes would facilitate personalized adoptive T-cell therapy.

To speed identification of tumor-reactive TCR genes, we developed a method for creating a library of randomly paired TCR genes in retroviral vectors. The TCR library was expressed in peripheral T cells for screening of antigen-specific TCR genes. We obtained TCR gene-engineered antitumor T cells within 2 weeks from frozen, stored specimens. We applied the method to identify tumor antigen-specific TCR α and β chain pairs from frozen tumor tissues and demonstrated the therapeutic potential of the library-derived TCR genes in a tumor xenograft model. We therefore demonstrate that antitumor TCR vectors can be obtained from small frozen tumor specimens without knowledge of the TCR sequences.

Materials and Methods

Specimens

All procedures in this study were approved by the Roswell Park Cancer Institute (RPCI)'s institutional review board and the institutional animal care and use committee. Informed consent was obtained from all patients. Ovarian tumor specimens were obtained at the surgery at RPCI and were frozen in liquid nitrogen and stored at −80°C. Generation of NY-ESO-1-specific T cells was performed as described previously (29). Total RNA from NY-ESO-1-specific T cells was obtained using TRI Reagent followed by Phenol/Chroloform extraction or the Direct-zol RNA MiniPrep kit (Zymo Research). Total RNA from tumor specimens (70–100 mg weight) was obtained by using tissue homogenizer in TRI Reagen followed by column purification with the Direct-zol RNA MiniPrep kit. Yield of total RNA was between 137–268 μg. Reverse transcription was performed using RevertAid First Strand cDNA Synthesis Kit using an oligo-dT primer (Thermo-Fisher) from 5 μg total RNA in a 20 μl reaction scale.

PCR amplification and purification of TCR variable regions

Primer sequences were listed in Supplementary Table S1. To enable unbiased PCR amplification of TCR-coding genes, typically, 2 μl cDNA from tumor tissues was mixed with multiplexed forward primers that are flanked by the common anchor sequences before the start codon (HTTCR#F for TCR α chain or HTTCR#C for TCR β chain) in separate tubes in 1×Phusion polymerase reaction master mix (Thermo-Fisher). A single cycle of 98°C for 40 seconds, rapid cooling to 72°C then slow (−0.1°C/second) cooling to 66°C, 66°C for 30 seconds and 72°C for 5 minutes was performed to synthesize the second-strand DNA. Unused multiplexed primers and all single-strand cDNA were destroyed by incubation with Exonuclease I at 37°C for 15 minutes followed by enzyme inactivation at 85°C for 15 minutes. To each reaction the forward primer which binds to the common anchor sequences in HTTCR#F and #C (HTTCR#D for TCR α chain or HTTCR#A for TCR β chain) and the reverse primer for the TCR constant region (HTTCR#E for TCR α chain or HTTCR#B for TCR β chain) was added in 1×Phusion polymerase reaction master mix. PCR was performed by 1 cycle of 98°C for 30 seconds; 2 cycles of 98°C for 10 seconds, 62°C for 30 seconds, and 72°C for 30 seconds; 30 cycles of 98°C for 10 seconds and 72°C for 60 seconds; and 1 cycle of 72°C for 2 minutes. The reaction was load on 1% agarose gel containing SYBR Safe DNA Gel Stain (Thermo Fisher Scientific) and electrophoresed at 90V for 30 minutes. The main band of TCR variable fragments at around 450 bp was excised under the transilluminator (Invitrogen) and DNA fragments were extracted using Zymoclean Gel DNA Recovery Kit (Zymo Research). DNA concentration was measured by absorbance at 260 nm. To amplify integrated TCR transgenes from T cells transduced with TCR genes, genomic DNA from TCR-transduced T cells was mixed with vector-specific primer pairs amplifying the entire TCR-expressing cassette (Forward: CGAATTCCCAAACTTAAGCTTGGTACCG; and Reverse: GCAGCGTATCCACATAGCGTAAAAGG) in 1×Phusion polymerase reaction mix. The PCR was performed by 1 cycle of 98°C for 30 seconds; 35 cycles of 98°C for 10 seconds, 71°C for 30 seconds, and 72°C for 40 seconds; and 1 cycle of 72°C for 2 minutes. Then, 1 μl of the reaction was mixed with Vβ-anchor-specific forward primer (HTTCR#A) and Cα-

specific reverse primer (HTTCR#E) in 1×Phusion polymerase reaction mix and cycled for 1 cycle of 98°C for 30 seconds; 35 cycles of 98°C for 10 seconds and 72°C for 70 seconds; and 1 cycle of 72°C for 2 minutes. Amplified DNA fragments were isolated and quantified as described above.

Assembling of TCR expressing cassette into a plasmid vector

The DNA fragment encoding cysteine-modified TCR Cβ-P2A fusion protein was amplified by PCR from a plasmid containing this fragment and purified by gel electrophoresis. Description of the destination plasmid vector is provided in Supplementary Fig. S1. Essentially, the destination plasmid was based on the MSCV retroviral vector. The splice acceptor site from the human elongation factor 1α promoter was introduced before the TCR cloning site. A residue in the TCR α chain constant region was mutated to a cysteine to enhance pairing with the cysteine-modified TCR β chain. The linearized destination plasmid (50 ng), which was treated with NotI and PspOMI and gel purified, was mixed with equimolar amounts of Vβ, Cβ-P2A (obtained by PCR using HTTCR#F and HTTCR#G primers from the destination plasmid), and Vα fragments in 10 μl 1×NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs) and incubated at 50°C for 60 minutes. To clone the TCR-expressing cassette that was amplified from genomic DNA of transduced T cells, linearized plasmid and TCR-expressing cassette insert were mixed at 1:2 molar ratio in 10 μl 1×NEBuilder HiFi DNA Assembly Master Mix and incubated at 50°C for 60 minutes. The assembled product was used to transform chemically competent *E. coli*, NEBStable, after purification by DNA Clean & Concentrator kit (Zymo Research). Transformed *E. coli* were spread over three 10cm agar plates and incubated 14–16 hours at 37°C. Confluent *E. coli* colonies in all three plates were pooled and plasmids were purified by ZymoPURE Plasmid Midiprep Kit (Zymo Research). Quality of this bulk plasmid preparation was examined by restriction enzyme treatment with NotI and PacI, which excise the TCR-expressing cassette from the plasmid backbone, followed by electrophoresis in an agarose gel. In some experiments, plasmids obtained from pooled *E. coli* colonies were used to re-transform competent *E. coli* to obtain single colonies. Some colonies were tested by DNA fingerprinting for TCR transgene by direct colony PCR using OneTaq (New England Biolabs) using a primer pair HTTCR#A and HTTCR#E; the reaction was then treated with AluI or MspI restriction enzyme (Thermo Scientific).

Retroviral transduction

Retroviral particles were produced by co-transfection of TCR-encoding transfer plasmids and pVSV-G envelope plasmids into the GP2-293 packaging cell line (Clontech) by Lipofectamine 2000 (Invitrogen-Thermo Scientific). Packaging cells were co-incubated with plasmids for 7 hours and culture medium was replaced. After 36 hours, supernatant was harvested, centrifuged for 5 minutes at 400×g for 5 minutes and immediately used for transduction of T cells. Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors' buffy coat using the density gradient method using lymphocyte separation medium and stored in a liquid nitrogen tank in 90% FBS plus 10% DMSO. PBMC were pre-activated by10 μg/ml phytohemagglutinin (PHA; Remel) for 40 hours in RPMI1640 medium supplemented with 10% FBS, Penicillin, Streptomycin and L-Glutamine in the presence of rhIL2 (10 U/ml, Sigma) rhIL7 (10 ng/ml, R&D Systems), and rhIL12p70 (20 ng/ml,

Peprotech). Typically, pre-activated PBMCs (1×10^5) were harvested, counted, and plated on 96-well flat-bottom plate precoated overnight with Retronectin (10 μg/ml) and monoclonal antibodies (mAbs) to human CD3 (5 μg/ml, OKT3; eBioscience) in the presence of rhIL2, rhIL7, and rhIL12. Typically, 125 μl retroviral supernatant was added to transduce T cells, which were then cultured for 24 hours. Cells were expanded in the presence of rhIL2 and rhIL7 without rhIL12 and used for evaluation within 7 days after transduction. Transduction of Jurkat (E6-1; ATCC) or J.RT3-T3.5 (ATCC) was performed similarly but without activating reagents and cytokines and using 6–12 μl retroviral supernatant.

Detection and isolation of antigen-specific T cells

NY-ESO-1-specific T cells were detected by specific MHC/peptide tetramer reagent (Ludwig Center for Cancer Research, University of Lausanne). TCR gene-transduced T cells were washed in PBS containing 1% FBS and incubated at 37°C for 15 minutes in the presence of phycoerythrin (PE)-conjugated tetramer (6 μg/ml) in 1% FBS-PBS. Cells were then stained by allophycocyanin (APC)-conjugated anti-CD4 and PerCP/Cy5.5-conjugated anti-CD8 (Biolegend) at 4°C for 15 minutes. Fluorescent signals were acquired by FACSCalibur instrument and analyzed by FlowJo software. In some experiments, tetramer$^+$ T cells were sorted using FACSAria instrument. Genomic DNA of sorted cells was obtained using Quick-gDNA MicroPrep kit (Zymo Research). Cytokine production from TCR gene-transduced T cells was tested by intracellular cytokine staining. Target cells were NY-ESO-1-expressing melanoma cell lines (HLA-A*02$^+$DRB1*01$^+$DPB1*04$^+$ SK-MEL-37; HLA-B*35$^+$ SK-MEL-52; HLA-DRB1*04$^+$DP*04$^+$ COLO 316 retrovirally transduced with NY-ESO-1 and CIITA genes) or NY-ESO-1-negative HLA-Cw*03$^+$ MZ-MEL-12. To test Cw*03-restricted NY-ESO-1-specific reactivity, a Cw*03-negative and NY-ESO-1-negative A2780 ovarian cancer cell line was engineered by the Sleeping Beauty transposon system. Cw*03 and NY-ESO-1 co-expressing transposon plasmid was constructed by inserting human elongation factor 1α promoter followed by Cw*03-P2A-EGFP(A206K)-T2A-NY-ESO-1 expressing cassette and the rabbit globin polyadenylation signal into pT2/BH (a gift from Perry Hackett: Addgene plasmid # 26556). A2780 was electroporated using the 4D-Nucleofector system (Lonza) with pCMV(CAT)T7-SB100 (a gift from Zsuzsanna Izsvak: Addgene plasmid # 34879) and pT2-EF-Cw3-GFP-ESO. GFP$^+$ clones were obtained by limiting dilution. To induce immunoproteasome expression, Cw*03$^+$NY-ESO-1$^+$ A2780 cells were pre-treated with 1,000 U/ml rhIFNγ (Peprotech) for 2 days. Before coculture with T cells, some aliquots of target cells were pulsed overnight with 10 μg/ml synthetic NY-ESO-1 peptide (Genscript) or 10 μg/ml recombinant NY-ESO-1 protein (Ludwig Institute for Cancer Research) and washed in RPMI1640 medium. T cells were cocultured with target cells for 6 hours in the presence of GolgiStop (BD Biosciences). Cells were stained by fluorescein isothiocyanate (FITC)-conjugated anti-CD4 and PerCP/Cy5.5-conjugated anti-CD8, permeabilized using BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences), and stained with PE-conjugated anti-TNFα and APC-conjugated anti-IFNγ (BioLegend). Cells were analyzed by FACSCalibur instrument and FlowJo software. SK-MEL-37 and SK-MEL-52 were obtained from the Ludwig Institute for Cancer Research (New York, NY). MZ-MEL-12 was obtained from Dr. Jäger (Krankenhaus Nordwest, Frankfurt Germany). Ovarian cancer cell lines (Colo 316 and A2780) were from our cell bank. Cancer cell lines were tested for mycoplasma contamination using the Mycoalert kit

(Lonza Walkersville). A2780 was certified to have no mycoplasma contamination by IDEXX BioResearch.

Tumor xenograft model

NOD/SCID/common γ chain-deficient (NSG) mice (Jackson Laboratory) were bred at the Laboratory Animal Resource at RPCI. Mice were inoculated with 1×10^6 IFNγ-treated $Cw3^+$NY-ESO-1^+ A2780. Therapeutic T cells were generated by retroviral transduction of the Cw*03-restricted NY-ESO-1(92–100)-specific secondary TCR library (Tumor #3). On day 3, mice received 4×10^6 TCR-transduced or untransduced T cells, or untreated. On days 3–5, all T cell-infused animals were intraperitoneally injected with 5×10^4 units IL2 (Peprotech). Tumor growth was measured every other day. Tumor volume was calculated by a formula 0.5×(longer diameter)×(shorter diameter)^2. Animals were sacrificed when tumor volume reached 2,000 mm^3.

Statistical analysis

Two-tailed paired or unpaired *t* test was used to evaluate statistically significant differences between the values in two groups. *P* values are annotated as follows: *, $P < 0.05$; and **, $P < 0.01$.

RESULTS

Amplification and cloning of TCR genes

A schematic TCR-expressing cassette designed for construction of retroviral vectors is shown in Fig. 1A. To enable stoichiometric expression of TCR α and β chains from a single transcript, TCR α and β chains were genetically connected via the P2A translational-skipping site (30, 31). The start codon for the TCR β chain was preceded by the Kozak consensus sequence (GCCACC) for efficient translation (32). The constant regions of both chains were modified by a cysteine residue to create an artificial disulfide bond which enhances paring of transgenic TCR α and β chains and inhibits pairing with endogenous TCRs (33). In the TCR-expressing cassette, the TCR α and β chain variable (Vα/Vβ), joining (Jα/Jβ) and the TCR β chain diverse (Dβ) regions that are critical for antigen recognition are required to be obtained from antigen-specific T cells. In contrast, the constant regions can be prepared as stocked fragments that contain artificial modifications such as cysteine modification and fusion to the P2A site. In addition, the TCR α chain constant region can be included in the destination plasmid vector to reduce the number of fragments to be assembled. A destination retroviral expression plasmid was constructed as shown in Supplementary Fig. S1A. The destination plasmid contains a TCR-expressing cassette in which the TCR Cα region was modified to contain a PspOMI-recognizing site (GGGCCC). The TCR-expressing cassette in the destination plasmid was excised by treatment with NotI and PspOMI restriction enzymes. The artificial PspOMI-recognizing site was corrected to the natural Cα sequence during the assembling reaction (Supplementary Fig. S1B).

The 5’ part of TCR α and β chains, Vα and Vβ, are highly variable. Therefore, without knowledge of TCR sequences, a large number of multiplexed forward primers are required

for PCR amplification. Two sets of multiplexed forward primers were designed for all known Vα and Vβ reported in the IMGT database (34). Sequences of primers used in this study are listed in Supplementary Table S1. The TCR Vβ-specific primer set consisted of 45 primers that are flanked with the common 5' sequence (the 3' region of the destination plasmid vector from the restriction enzyme [NotI] site; designated as Vec' in Fig. 1B) before the start codon. Similarly, 49 Vα-specific primers had another common sequence (the 3' region of the P2A site). These common flanked sequences serve as anchors for PCR amplification by the common primer sets. The procedures for amplification and cloning of TCR genes are depicted in Fig. 1B. In all experiments for this study, we used cDNA prepared from total RNA using oligo dT primers and a reverse transcriptase (Step 1). Because PCR amplification by multiplexed primers can cause an amplification bias due to different efficiencies for each primer, amplification of Vα and Vβ regions was performed using the anchor-specific forward primers which extend the 5' anchor by flanked sequence (Vec" in Fig. 1B) and reverse primers specific for the TCR constant region (Cα and Cβ). To end this, the second-strand TCR cDNA was synthesized by a single-cycle polymerase reaction primed by multiplexed primers, thereby adding an anchor sequence to the 5' end of the second-strand cDNA (Step 2), followed by elimination of excess primers by Exonuclease I treatment (Step 3). Then, Vα and Vβ fragments were amplified by PCR using an anchor-specific forward primer and and a reverse primer specific for the TCR common region (Cβ or Cα) (Step 4). The 5' region of the resulting Vβ fragment has a 30-nucleotide overlap with the 3' region from a NotI cloning site in the destination plasmid vector, whereas the Vα fragment has an overlap with the 3' region of P2A-sequence. The cysteine-modified constant region for TCR β chain linked to the P2A-sequence was PCR-amplified from the destination plasmid containing this fragment. Three fragments, VDJβ, Cβ-P2A, and VJα, were assembled with a linearized destination retroviral plasmid vector containing the cysteine-modified Cα fragment by a modified Gibson assembly using NEBuilder HiFi DNA Assembly Master Mix (Step 5). Using frozen stocked Cβ-P2A and linearized destination vector fragments, we prepared assembled vectors for transformation of competent *E. coli* cells within 4 hours.

Construction of TCR-expressing retroviral vectors from tumor antigen-specific T-cell clones

To validate the rapid TCR-cloning method, we constructed TCR-expressing retroviral vectors from established T-cell clones that have unique combinations of TCR α and β chain genes. Four $CD8^+$ T-cell clones ("AL": HLA-A*02-restricted NY-ESO-1(157–165)-specific; "JD": HLA-A*02-restricted NY-ESO-1(157–165)-specific; "KQ": HLA-B*35-restricted NY-ESO-1(94–102)-specific; and "PP": HLA-B*35-restricted NY-ESO-1(94–104)-specific) and five $CD4^+$ T-cell clones ("SB": HLA-DR*01-restricted NY-ESO-1(95–106)-specific; "JM": HLA-DP*04-restricted NY-ESO-1(157–170)-specific; "3B5": HLA-DP*04-restricted NY-ESO-1(157–170)-specific; "5B8": HLA-DP*04-restricted NY-ESO-1(157–170)-specific; and "PB-T": HLA-DR*04-restricted NY-ESO-1(111–143)-specific) were selected for experiments All T-cell clones were established from peripheral blood or tumor specimens obtained from ovarian cancer patients at RPCI as described (30, 35, 36). As a control T-cell clone, the Jurkat T-lymphoma cell line (ATCC; TIB-152) was included. Vβ and Vα fragments were amplified as a single band for all T-cell clones tested (data not

shown). The assembled TCR-expressing vectors were used to transform chemically competent *E. coli*. Transformed cells were spread and incubated overnight on agar plates to produce confluent colonies. To confirm that the TCR-expressing cassette was correctly assembled, bulk plasmids that were obtained from pooled colonies were digested by NotI and PacI restriction enzymes, which excise full-length TCR-expressing cassettes (Fig. 1B). A single band with the expected size for the TCR-expressing cassette was excised from the plasmid at around 1.8kb (data not shown), indicating that our cloning procedures correctly assembled fragments as the expressing cassette.

To test the functionality of the cloned TCR, healthy donor T cells were polyclonally activated and infected with retroviruses generated from bulk plasmids containing T-cell clone-derived TCR-expressing cassettes. After a single infection, 25–35% of T cells expressed transduced TCR, as determined by the increase in TCR Vβ subtype expression where appropriate antibodies were available (Vβ16 for JD-TCR and Vβ5.3 for KQ-TCR) (Fig. 2A). Functional expression of antigen-specific TCR α and β chain pairs was determined by MHC/peptide tetramer staining. Staining of untransduced and irrelevant TCR gene-transduced T cells was negligible. All four HLA class I-restricted TCR expressed on $CD8^+$ T cells were stained by corresponding tetramers (HLA-A*02/NY-ESO-1(157–165) for AL and JD, HLA-B*35/NY-ESO-1(94–102) for KQ, or HLA-B*35/NY-ESO-1 (94–104) for PP) (Fig. 2B and Supplementary Fig. S2). TCR-transduced T cells produced both IFNγ and TNFα upon coculturing with the antigen and HLA-expressing cancer cell lines (Fig. 2C). To assess functional expression of HLA class II-restricted NY-ESO-1-specific TCRs for which MHC/peptide-tetramer reagents were not available, TCR gene-transduced T cells were cocultured with antigen-pulsed target cells or MHC class II and NY-ESO-1-coexpressing cancer cells followed by intracellular cytokine staining. As shown in Supplementary Fig. S3, HLA class II-restricted TCR gene-transduced T cells produced IFNγ and TNFα upon antigen stimulation. These results demonstrate that our cloning protocol constructs functional TCR-expressing vectors for all T-cell clones tested without the need of TCR sequence information.

Construction and characterization of randomly paired TCR libraries from polyclonal T cells

Next, we applied our TCR-cloning protocol to construct randomly paired TCR-expression libraries from polyclonal T-cell populations. We first tested the feasibility of constructing TCR-expression libraries from polyclonal T cells from PBMC of three healthy donors. Both TCR Vα and Vβ were amplified from PBMC cDNA (data not shown). Assembled plasmids were amplified in competent cells and purified from pooled colonies. The size of the library from one 50 μl vial of the competent cells was determined to be $2.8 \pm 0.5 \times 10^5$ (Mean ± SD for 3 donors) by serial dilution of the transformed cells.

To investigate whether TCR gene amplification using the common primer sets against anchor-specific forward and common TCR constant region-specific reverse primers enables unbiased amplification of different TCR species, we compared usage of TCR Vβ subtypes in $CD3^+$ T cells in PBMC and plasmid library by flow cytometry using Vβ subtype-specific antibodies. TCR Vβ usage in the TCR-expressing retroviral library was investigated by infecting retroviral particles into a Jurkat T-lymphoma subline with a mutated TCR β chain

(J.RT3-T3.5 [J.RT3]; ATCC: TIB-153). J.RT3 was transduced with retroviral library at suboptimal viral titers that transduce less than 30% of cells to minimize multi-copy transduction. Expression of cell-surface TCR Vβ was tested by flow cytometry using 24 different antibodies against Vβ subtypes. Fig. 3A shows the relationship between the frequency of each Vβ subtype in $CD3^+$ T cells in PBMC and library-transduced J.RT3 for 3 independent libraries from different donors. Overall, Vβ usage in $CD3^+$ T cells was retained in the library. Fig. 3B compares mean percentages of Vβ usage with results of statistical analyses by paired *t* tests. Although there were a few significant differences in the Vβ usage between PBMC and TCR library, such as overrepresentation of Vβ5.1 in the library, and several minor differences, the Vβ usage in peripheral T cells was well reproduced in the library. These results suggest that the majority of TCR-gene species in the polyclonal T-cell population were PCR-amplified and assembled without bias.

Recovery of correctly paired TCR from randomly paired TCR-expression libraries

Next, we tested whether our protocol could identify a tumor antigen-specific TCR gene from a polyclonal T-cell population without isolating tumor antigen-specific T cells. Immunogenic tumors are enriched in tumor antigen-specific T cells (29). Therefore, we hypothesized that randomly assembled TCR libraries from tumor specimens could be used to identify tumor antigen-specific TCR genes (Fig. 4A).

Even though tumors are enriched with tumor antigen-specific T cells, the probability that correct tumor antigen-specific TCR pairs would form through random pairing is low. Therefore, we tested the feasibility of recovering correctly paired tumor antigen-specific TCR from randomly paired TCR library in two ways. First, we mixed the HLA-A*02-restricted NY-ESO-1(157–165)-specific TCR-expressing plasmid (AL-TCR) with an irrelevant TCR-expressing plasmid at varying ratios (10%, 1%, 0.1% and 0.01% for AL-TCR-expressing plasmid). Retroviral vectors were produced using mixed plasmids and were used to infect activated T cells from a healthy donor. As expected, specific TCR-expressing T cells as detected by a HLA-A*02/NY-ESO-1(157–165) tetramer decreased as the ratio of the AL-TCR-expressing plasmid decreased and became nearly undetectable when that ratio was at and below 0.1% (Supplementary Fig. S4). To isolate the NY-ESO-1-specific TCR-expressing transgene, the tetramer-stained T cells were sorted by flow-cytometry and genomic DNA with the integrated retroviral TCR transgene was extracted. The TCR transgene was amplified by nested PCR of genomic DNA and was re-assembled into a TCR-expressing plasmid. As expected, we obtained sufficient number (358 cells) of tetramer-stained cells from 1% AL-TCR mixture from 6×10^5 transduced T cells, whereas only 27 and 4 cells were obtained from 0.1% and 0.01% mixtures, respectively. Furthermore, although nested PCR yielded a product of the expected size from 27 tetramer-sorted cells from the 0.1% mixture, it failed to recover the transgene from 4 cells from the 0.01% mixture. As shown in Supplementary Fig. S4, all vectors generated by re-assembling of nested PCR products in the TCR-expressing plasmids similarly transduced A*02-restricted NY-ESO-1(157–165)-specific TCR. These results demonstrated that even though frequencies of correctly paired TCRs in the primary library are as low as 0.1%, tumor antigen-specific TCR genes can be identified by isolation of TCR-transduced cells with desired functions, such as tetramer binding, and PCR amplification of integrated TCR

transgenes by nested PCR. Assuming unbiased PCR amplification and assembly of TCR α and β chain fragments, 0.1% of a correctly paired TCR-expressing plasmid should be present in the library when a specific T-cell clone is present at 3.3% among total TCRαβ-expressing T cells.

Next, we tested whether we could recover correctly paired TCR from cDNA from the same AL clone and an irrelevant T-cell clone mixed at varying ratios (30%, 9%, 2.7%, and 0.8% for AL cDNA). Randomly paired TCR-expressing libraries were prepared from the cDNA mixtures and T cells were transduced by retroviral libraries. As described for plasmid mixtures, we stained transduced T cells with a specific tetramer (Fig. 4B), sorted tetramer-stained T cells, and amplified TCR-expressing transgenes to clone into the retroviral plasmid. As shown in Fig. 4C, correctly paired antigen-specific TCR was recovered from the 2.7% AL cDNA mixture, whereas nested PCR of tetramer sorted cells (8 cells from 0.6×10^6 transduced T cells) from 0.8% AL cDNA mixture failed to amplify the TCR transgene. Therefore, in accordance with the experiments using plasmid mixtures, we expected that correctly paired antigen-specific TCR could be recovered when antigen-specific T-cell clones are present at or above 3% among total TCRαβ-expressing T cells in the specimen.

Identification of tumor antigen-specific TCR from tumor-derived TCR-expression library

Using our TCR amplification and assembly protocol described above, we constructed a TCR gene library from 3 frozen tumor specimens that were known to be infiltrated by high frequency HLA-Cw*03-restricted NY-ESO-1-specific $CD8^+$ T cells (29). Percentages of Cw*03/NY-ESO-1(92–100) tetramer-stained cells among $CD8^+$ T cells were 20.9%, 26.2%, and 16.4% for tumor #1, #2, and #3, respectively. However, percentages of tetramer-stained cells among total $CD3^+$ T cells were not available. TCR variable fragments were amplified from cDNA of frozen ovarian tumor specimens and assembled as retroviral plasmid vectors. Then, retroviral particles were produced using the bulk plasmid library.

Polyclonally activated T cells were infected once with the tumor-derived TCR-expression library at suboptimal viral titers to minimize multi-copy transduction. As shown in Fig. 4D, transduction with libraries derived from tumors #2 and #3 only slightly increased tetramer-stained cells over background staining, and transduction with the library derived from tumor #1 induced no detectable tetramer-stained cells. The secondary TCR library was prepared using nested PCR products from tetramer-sorted T cells and was used to prepare retroviral vectors to transduce primary T cells. A large fraction of the tumor #2 and #3-derived secondary TCR library-transduced T cells were reactive to the cognate antigen, as demonstrated by specific tetramer staining (Fig. 5A) and cytokine release against peptide-pulsed target cells (Fig. 5B). In contrast, the tumor #1-derived library did not contain tetramer-reactive TCRs (data not shown). TCR clones in the secondary library were characterized by TCR transgene DNA fingerprinting by digestion with restriction enzymes. As shown Supplementary Fig. S5, the secondary TCR library for tumor #2 was significantly enriched by a single clone (7/14 clones), whereas tumor #3 was enriched with 2 clones (clonotype 3A: 7/14 and clonotype 3B: 4/14). Sequences for selected clones for each clonotype were shown in Supplementary Table S2. Clonotypes 3A and 3B share the same TCR β chain but have unrelated α chains. Transduction with the enriched TCR clone from

tumor #2 and the clonotype 3A but not 3B from tumor #3 induced tetramer$^+$ T cells (Supplementary Fig. S6). It is unclear why clonotype 3B was enriched in the secondary library but did not bind tetramer.

In contrast to TCRs obtained from established T-cell clones or single cell-based approaches, our strategy based on random pairing could generate artificial TCR pairs that could cross-react to self-antigens and cause toxicity in patients. In order to determine specificity of library-derived TCRs, we tested recognition of Glycine-substituted peptides for the Cw*03-binding NY-ESO-1(92–100: LAMPFATPM) epitope. As shown in Supplementary Fig. S7, substitution of any amino acid residue in the central part (MPFATPM) of the epitope almost completely abrogated recognition by both tumor #2- and #3-derived TCRs, indicating NY-ESO-1-specificity. *In silico* search for the TCR-recognizing motif (MPFATPM) through the ScanProsite tool (http://prosite.expasy.org/scanprosite/) found that only NY-ESO-1 has this motif among mammalian proteins.

We next investigated if a newly identified TCR could be functional and capable of rejecting tumors. We utilized an *in vivo* tumor xenograft model to study the therapeutic effect of the tumor #3-derived secondary TCR library-transduced T cells. Consistent with a report that the NY-ESO-1(92–100) epitope is generated by immunoproteasome (37), *in vitro* recognition of the A2780 ovarian cancer cell line (which was engineered to express Cw*03 and NY-ESO-1) by engineered T cells was significantly enhanced by IFNγ treatment of cancer cells (Fig. 5C). NSG mice were inoculated subcutaneously with HLA-Cw*03$^+$NY-ESO-1$^+$ A2780. On day 3, when palpable tumor was established, mice were intravenously infused with 4×10^6 T cells either untransduced or transduced with the tumor #3 TCR library. Whereas untransduced T cells showed no antitumor effects, TCR gene-transduced T-cell products eliminated established tumors in all animals (Fig. 5D).

Discussion

Adoptive transfer of autologous tumor antigen-specific T cells is an effective therapeutic treatment for cancer patients (38, 39). Gene engineering of patients' peripheral T cells with tumor antigen-specific TCR or chimeric antigen receptor (CAR) gene can overcome challenges in obtaining sufficient numbers of tumor antigen-specific T cells from patients' specimens (16). In solid tumors, because most known tumor-specific antigens are intracellular proteins, such as cancer-testis antigens, TCR rather than CAR genes may be more suitable for manufacture of therapeutic T-cell products. However, because of the variety of HLA types and tumor antigen expression patterns, a large panel of TCR genes specific for different tumor antigens and HLA restriction elements would be required for the treatment of a population of patients with different tumor types. To accelerate the discovery of tumor antigen-specific TCR α and β chain pairs, we have developed a method to construct a library of randomly paired TCRs from tumor tissues that are infiltrated by tumor antigen-specific T cells.

In contrast to other methods that require single-cell suspension, our method only requires snap frozen tumor specimens, which can be prepared at most clinical sites. Furthermore, as this protocol requires only a few micrograms of total RNA from tumor specimens, we expect

that the protocol could be applied to small specimens such as needle biopsies from which isolation of infiltrating T cells may be challenging. Following standard procedures of total RNA extraction and reverse transcription, variable regions of TCR α and β chains were amplified by the common primers, but not TCR Vα or Vβ-specific multiplexed primers, in order to minimize PCR bias. These variable fragments were assembled as expressing cassettes and cloned. Through our method, a tumor-infiltrating T cell-derived TCR library can be prepared as retroviral expression plasmids within 24-hours. We then retrovirally transduced the TCR library into peripheral T cells, in order to screen for tumor antigen-specific TCRs. We successfully identified tumor antigen-specific TCR pairs from 2 out of 3 frozen ovarian tumor specimens. The TCRs obtained by this approach were functional and able to recognize tumor *in vitro*. We demonstrated the therapeutic potential of one of the library-derived TCRs (Tumor #3) by adoptively transferring the T cells in a tumor xenograft model.

Our method identified tumor-reactive TCRs despite low frequencies of T cells infected by tumor-derived TCR-expression library at suboptimal viral titers. One of the tumor-derived libraries (from tumor #1) did not contain many tumor-reactive TCRs, although the original tumor specimen contained a high frequency of tetramer-reactive $CD8^+$ T cells (data not shown). It is possible that the specimen that was used for single-cell suspension for T-cell staining and that for RNA extraction contained different fractions of tetramer-reactive T cells. Alternatively, tetramer-reactive T cells could be composed of oligoclonal populations, for which the probability to form functional TCR pairs in the library exponentially decreases. Although the multiplexed primers were designed to amplify all known TCR variable regions, it may be possible that the tetramer-reactive TCR gene had unknown polymorphic mutations in their primer-binding regions. Here we used a small library (3×10^5) to screen on a limited scale. Although this library size is considered to be sufficient to obtain correct TCR pairs from a T-cell clone of more than 1% frequency among total T cells, the library size could be expanded by the use of electro-competent cells to identify specific TCR pairs from less frequent T-cell clones, for example, those in peripheral blood.

Randomly paired libraries of immunoglobulin heavy and light chains have been used to identify antibodies against therapeutic targets including cancer (40). In general, immunoglobulin heavy and light chains are PCR-amplified and randomly fused via a linker peptide to generate single-chain variable fragments. Then, pairs with the desired specificity are isolated by screening the library for binding to target antigens. However, this method has not been tested to identify antigen-specific TCR heterodimer genes. Our results demonstrated that a tumor-derived randomly paired TCR library is a useful resource to identify tumor antigen-specific TCR pairs. In addition, we were able to quickly generate viral vector constructs containing the new TCRs, which not only sped up the screening process, but provided a tool to genetically engineer T cells for adoptive transfer studies.

In contrast to other methods used to obtain paired TCR α and β chain sequences from a single cell by single-cell PCR, emulsion PCR, or the pairSEQ platform, our method has the possibility of generating artificial TCR pairs that recognized cancer targets with higher affinity than the natural tumor antigen-specific TCR pair (41). Artificial TCR pairs can cross-react with unexpected antigens, including some expressed in normal tissues. Off-target

toxicity of the TCR gene-engineered T cell product is a problem with non-natural TCRs such as affinity-enhanced TCRs or murine TCRs (42–44). The TCRs we identified here from tumor specimens are NY-ESO-1-specific, as determined by the TCR-recognizing motif using Glycine-substituted peptides. Although testing off-target reactivity is important for any therapeutic TCR gene, candidates for therapeutic TCR genes identified by our method can be extensively tested for cross-reactivity against a panel of normal tissues and genes that contain homologous sequences of the TCR epitope.

In summary, we established a method for discovery and identification of relevant TCRs that can be utilized in a viral vector construct form for downstream translational validation towards therapeutic adoptive cell therapy for cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We appreciate technical supports from E. Cheryl and AJ. François. We thank members of Center for Immunotherapy at RPCI for valuable comments.

Grant Support

This work was supported by RPCI, National Cancer Institute (NCI) grants P30CA016056 and P50CA159981, Roswell Park Alliance Foundation, Ovarian Cancer Research Fund Alliance (327679) and the New York State Stem Cell Science (NYSTEM: C030158).

References

1. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science. 2002; 298:850–4. [PubMed: 12242449]
2. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin Cancer Res. 2011; 17:4550–7. [PubMed: 21498393]
3. Lu YC, Yao X, Li YF, El-Gamil M, Dudley ME, Yang JC, et al. Mutated PPP1R3B is recognized by T cells used to treat a melanoma patient who experienced a durable complete tumor regression. J Immunol. 2013; 190:6034–42. [PubMed: 23690473]
4. Bollard CM, Aguilar L, Straathof KC, Gahn B, Huls MH, Rousseau A, et al. Cytotoxic T lymphocyte therapy for Epstein-Barr virus+ Hodgkin's disease. J Exp Med. 2004; 200:1623–33. [PubMed: 15611290]
5. Vignard V, Lemercier B, Lim A, Pandolfino MC, Guilloux Y, Khammari A, et al. Adoptive transfer of tumor-reactive Melan-A-specific CTL clones in melanoma patients is followed by increased frequencies of additional Melan-A-specific T cells. J Immunol. 2005; 175:4797–805. [PubMed: 16177129]
6. Mackensen A, Meidenbauer N, Vogl S, Laumer M, Berger J, Andreesen R. Phase I study of adoptive T-cell therapy using antigen-specific CD8+ T cells for the treatment of patients with metastatic melanoma. J Clin Oncol. 2006; 24:5060–9. [PubMed: 17075125]
7. Stevanovic S, Draper LM, Langhan MM, Campbell TE, Kwong ML, Wunderlich JR, et al. Complete regression of metastatic cervical cancer after treatment with human papillomavirus-targeted tumor-infiltrating T cells. J Clin Oncol. 2015; 33:1543–50. [PubMed: 25823737]

8. Tsuji T, Yasukawa M, Matsuzaki J, Ohkuri T, Chamoto K, Wakita D, et al. Generation of tumor-specific, HLA class I-restricted human Th1 and Tc1 cells by cell engineering with tumor peptide-specific T-cell receptor genes. Blood. 2005; 106:470–6. [PubMed: 15790789]
9. Johnson LA, Heemskerk B, Powell DJ Jr, Cohen CJ, Morgan RA, Dudley ME, et al. Gene transfer of tumor-reactive TCR confers both high avidity and tumor reactivity to nonreactive peripheral blood mononuclear cells and tumor-infiltrating lymphocytes. J Immunol. 2006; 177:6548–59. [PubMed: 17056587]
10. Clay TM, Custer MC, Sachs J, Hwu P, Rosenberg SA, Nishimura MI. Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. J Immunol. 1999; 163:507–13. [PubMed: 10384155]
11. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. Science. 2006; 314:126–9. [PubMed: 16946036]
12. Chodon T, Comin-Anduix B, Chmielowski B, Koya RC, Wu Z, Auerbach M, et al. Adoptive Transfer of MART-1 T-Cell Receptor Transgenic Lymphocytes and Dendritic Cell Vaccination in Patients with Metastatic Melanoma. Clin Cancer Res. 2014; 20:2457–65. [PubMed: 24634374]
13. Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood. 2009; 114:535–46. [PubMed: 19451549]
14. Rapoport AP, Stadtmauer EA, Binder-Scholl GK, Goloubeva O, Vogl DT, Lacey SF, et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. Nat Med. 2015; 21:914–21. [PubMed: 26193344]
15. Debets R, Donnadieu E, Chouaib S, Coukos G. TCR-engineered T cells to treat tumors: Seeing but not touching? Semin Immunol. 2016; 28:10–21. [PubMed: 26997556]
16. June CH, Riddell SR, Schumacher TN. Adoptive cellular therapy: a race to the finish line. Sci Transl Med. 2015; 7:280ps7.
17. Wang GC, Dash P, McCullers JA, Doherty PC, Thomas PG. T cell receptor alphabeta diversity inversely correlates with pathogen-specific antibody levels in human cytomegalovirus infection. Sci Transl Med. 2012; 4:128ra42.
18. Kobayashi E, Mizukoshi E, Kishi H, Ozawa T, Hamana H, Nagai T, et al. A new cloning and expression system yields and validates TCRs from blood lymphocytes of patients with cancer within 10 days. Nat Med. 2013; 19:1542–6. [PubMed: 24121927]
19. Seitz S, Schneider CK, Malotka J, Nong X, Engel AG, Wekerle H, et al. Reconstitution of paired T cell receptor alpha- and beta-chains from microdissected single cells of human inflammatory tissues. Proc Natl Acad Sci USA. 2006; 103:12057–62. [PubMed: 16882720]
20. Dossinger G, Bunse M, Bet J, Albrecht J, Paszkiewicz PJ, Weissbrich B, et al. MHC multimer-guided and cell culture-independent isolation of functional T cell receptors from single cells facilitates TCR identification for immunotherapy. PLoS One. 2013; 8:e61384. [PubMed: 23637823]
21. Han A, Glanville J, Hansmann L, Davis MM. Linking T-cell receptor sequence to functional phenotype at the single-cell level. Nat Biotechnol. 2014; 32:684–92. [PubMed: 24952902]
22. Turchaninova MA, Britanova OV, Bolotin DA, Shugay M, Putintseva EV, Staroverov DB, et al. Pairing of T-cell receptor chains via emulsion PCR. Eur J Immunol. 2013; 43:2507–15. [PubMed: 23696157]
23. Munson DJ, Egelston CA, Chiotti KE, Parra ZE, Bruno TC, Moore BL, et al. Identification of shared TCR sequences from T cells in human breast cancer using emulsion RT-PCR. Proc Natl Acad Sci USA. 2016; 113:8272–7. [PubMed: 27307436]
24. Linnemann C, Heemskerk B, Kvistborg P, Kluin RJ, Bolotin DA, Chen X, et al. High-throughput identification of antigen-specific TCRs by TCR gene capture. Nat Med. 2013; 19:1534–41. [PubMed: 24121928]
25. Gros A, Parkhurst MR, Tran E, Pasetto A, Robbins PF, Ilyas S, et al. Prospective identification of neoantigen-specific lymphocytes in the peripheral blood of melanoma patients. Nat Med. 2016; 22:433–8. [PubMed: 26901407]

26. Pasetto A, Gros A, Robbins PF, Deniger DC, Prickett TD, Matus-Nicodemos R, et al. Tumor- and Neoantigen-Reactive T-cell Receptors Can Be Identified Based on Their Frequency in Fresh Tumor. Cancer Immunol Res. 2016; 4:734–43. [PubMed: 27354337]
27. Howie B, Sherwood AM, Berkebile AD, Berka J, Emerson RO, Williamson DW, et al. High-throughput pairing of T cell receptor alpha and beta sequences. Sci Transl Med. 2015; 7:301ra131.
28. Padovan E, Casorati G, Dellabona P, Meyer S, Brockhaus M, Lanzavecchia A. Expression of two T cell receptor alpha chains: dual receptor T cells. Science. 1993; 262:422–4. [PubMed: 8211163]
29. Matsuzaki J, Gnjatic S, Mhawech-Fauceglia P, Beck A, Miller A, Tsuji T, et al. Tumor-infiltrating NY-ESO-1-specific CD8+ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. Proc Natl Acad Sci USA. 2010; 107:7875–80. [PubMed: 20385810]
30. Matsuzaki J, Tsuji T, Luescher IF, Shiku H, Mineno J, Okamoto S, et al. Direct tumor recognition by a human CD4(+) T-cell subset potently mediates tumor growth inhibition and orchestrates anti-tumor immune responses. Sci Rep. 2015; 5:14896. [PubMed: 26447332]
31. Banu N, Chia A, Ho ZZ, Garcia AT, Paravasivam K, Grotenbreg GM, et al. Building and optimizing a virus-specific T cell receptor library for targeted immunotherapy in viral infections. Sci Rep. 2014; 4:4166. [PubMed: 24566718]
32. Kozak M. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. 1987; 15:8125–48. [PubMed: 3313277]
33. Cohen CJ, Li YF, El-Gamil M, Robbins PF, Rosenberg SA, Morgan RA. Enhanced antitumor activity of T cells engineered to express T-cell receptors with a second disulfide bond. Cancer Res. 2007; 67:3898–903. [PubMed: 17440104]
34. Lefranc MP, Giudicelli V, Duroux P, Jabado-Michaloud J, Folch G, Aouinti S, et al. IMGT(R), the international ImMunoGeneTics information system(R) 25 years on. Nucleic Acids Res. 2015; 43:D413–22. [PubMed: 25378316]
35. Odunsi K, Qian F, Matsuzaki J, Mhawech-Fauceglia P, Andrews C, Hoffman EW, et al. Vaccination with an NY-ESO-1 peptide of HLA class I/II specificities induces integrated humoral and T cell responses in ovarian cancer. Proc Natl Acad Sci USA. 2007; 104:12837–42. [PubMed: 17652518]
36. Tsuji T, Matsuzaki J, Caballero OL, Jungbluth AA, Ritter G, Odunsi K, et al. Heat shock protein 90-mediated peptide-selective presentation of cytosolic tumor antigen for direct recognition of tumors by CD4(+) T cells. J Immunol. 2012; 188:3851–8. [PubMed: 22427632]
37. Woods K, Knights AJ, Anaka M, Schittenhelm RB, Purcell AW, Behren A, et al. Mismatch in epitope specificities between IFNgamma inflamed and uninflamed conditions leads to escape from T lymphocyte killing in melanoma. J Immunother Cancer. 2016; 4:10. [PubMed: 26885372]
38. Rosenberg SA. Cell transfer immunotherapy for metastatic solid cancer--what clinicians need to know. Nat Rev Clin Oncol. 2011; 8:577–85. [PubMed: 21808266]
39. Yang F, Jin H, Wang J, Sun Q, Yan C, Wei F, et al. Adoptive Cellular Therapy (ACT) for Cancer Treatment. Adv Exp Med Biol. 2016; 909:169–239. [PubMed: 27240459]
40. Sanchez-Martin D, Sorensen MD, Lykkemark S, Sanz L, Kristensen P, Ruoslahti E, et al. Selection strategies for anticancer antibody discovery: searching off the beaten path. Trends Biotechnol. 2015; 33:292–301. [PubMed: 25819764]
41. Nakatsugawa M, Yamashita Y, Ochi T, Tanaka S, Chamoto K, Guo T, et al. Specific roles of each TCR hemichain in generating functional chain-centric TCR. J Immunol. 2015; 194:3487–500. [PubMed: 25710913]
42. Morgan RA, Chinnasamy N, Abate-Daga D, Gros A, Robbins PF, Zheng Z, et al. Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. J Immunother. 2013; 36:133–51. [PubMed: 23377668]
43. Linette GP, Stadtmauer EA, Maus MV, Rapoport AP, Levine BL, Emery L, et al. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. Blood. 2013; 122:863–71. [PubMed: 23770775]
44. Cameron BJ, Gerry AB, Dukes J, Harper JV, Kannan V, Bianchi FC, et al. Identification of a Titin-derived HLA-A1-presented peptide as a cross-reactive target for engineered MAGE A3-directed T cells. Science Tansl Med. 2013; 5:197ra03.

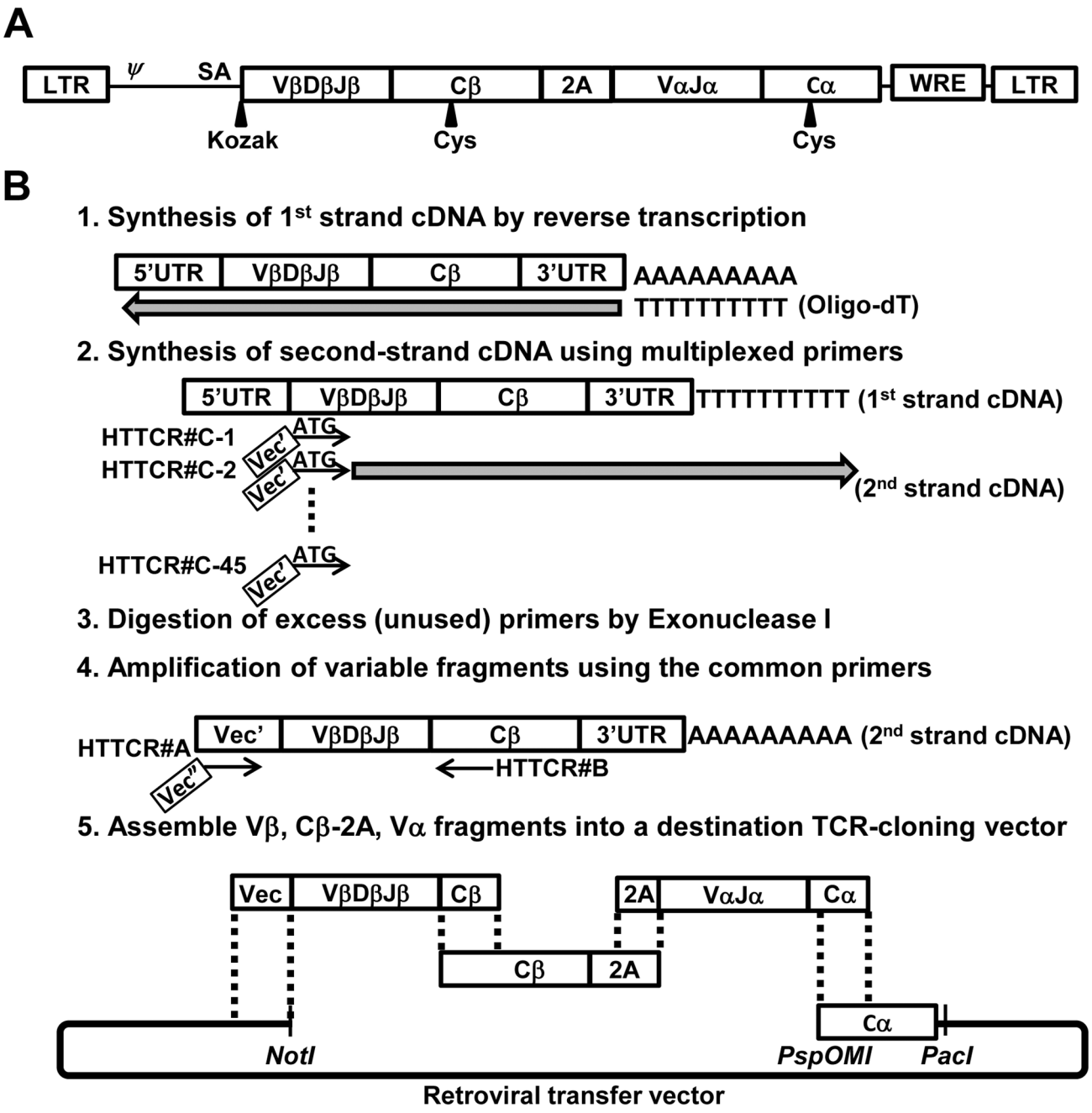

Figure 1.

Amplification of TCR genes and cloning into retroviral plasmid vectors. **A,** Schematic representation of the TCR-expressing cassette. Abbreviations used are: LTR, long terminal repeats; *ψ*, packaging signal; SA, splice acceptor site; and WRE: Woodchuck hepatitis virus posttranscriptional regulatory element. **B,** TCR amplification and cloning procedures. Detailed procedures are described in the materials and methods section. Vec' indicates anchor sequence to provide common sequence for amplification by the common primer sets. Anchor sequence is extended by Vec" during PCR amplification to provide 30 nucleotide overlap (designated as Vec) with the destination plasmid. Steps 1–4 were shown only for

TCR β chain for simplicity. For the α chain, steps 2–4 are performed in a separate tube using different primers: HTTCR#F in step 2 and HTTCR#D and #E in step 4.

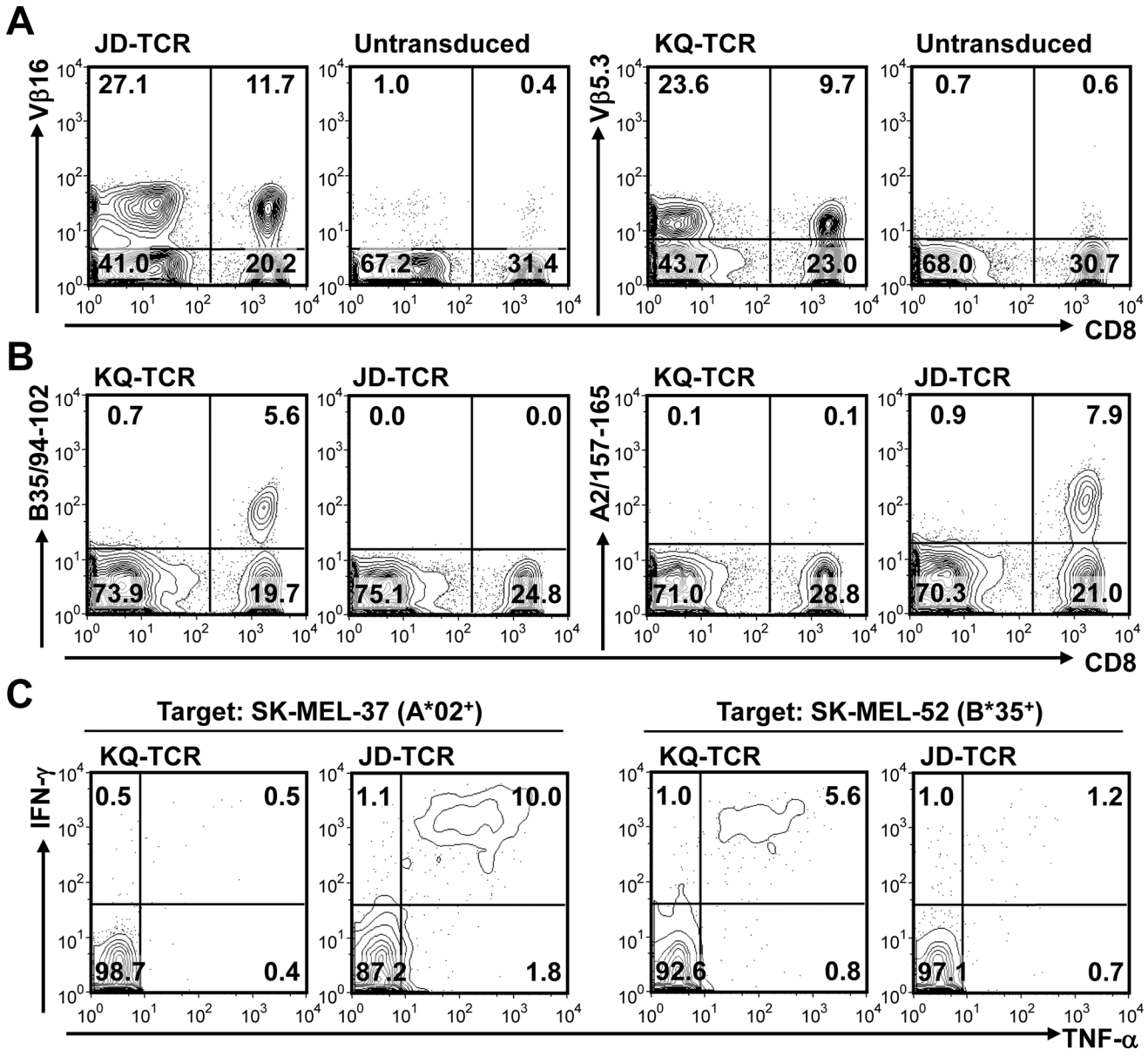

Figure 2.

Construction of TCR-expressing retroviral vectors from tumor antigen-specific T-cell clones. **A**, Expression of the TCR transgene after retroviral transduction. Bulk plasmids were used for production of retroviral particles. Polyclonally activated T cells that were transduced by retroviral JD-TCR (Vβ16) or KQ-TCR (Vβ5.3)-expressing vectors were stained by Vβ subtype-specific antibodies. **B,** Binding of TCR gene-transduced T cells to a specific MHC/peptide tetramer. HLA B*35-restricted NY-ESO-1(94–102) peptide-specific KQ-TCR and HLA A*02-restricted NY-ESO-1(157–165) peptide-specific JD-TCR transduced T cells were stained with the corresponding tetramers and analyzed by flow cytometry. **C,** Recognition of NY-ESO-1-expressing cancer cell lines. TCR gene-transduced T cells were cocultured with cancer cells for 6 hours in the presence of Monensin. Expression of IFNγ

and TNFα was examined by intracellular staining. Each experiment was performed at least twice with similar results.

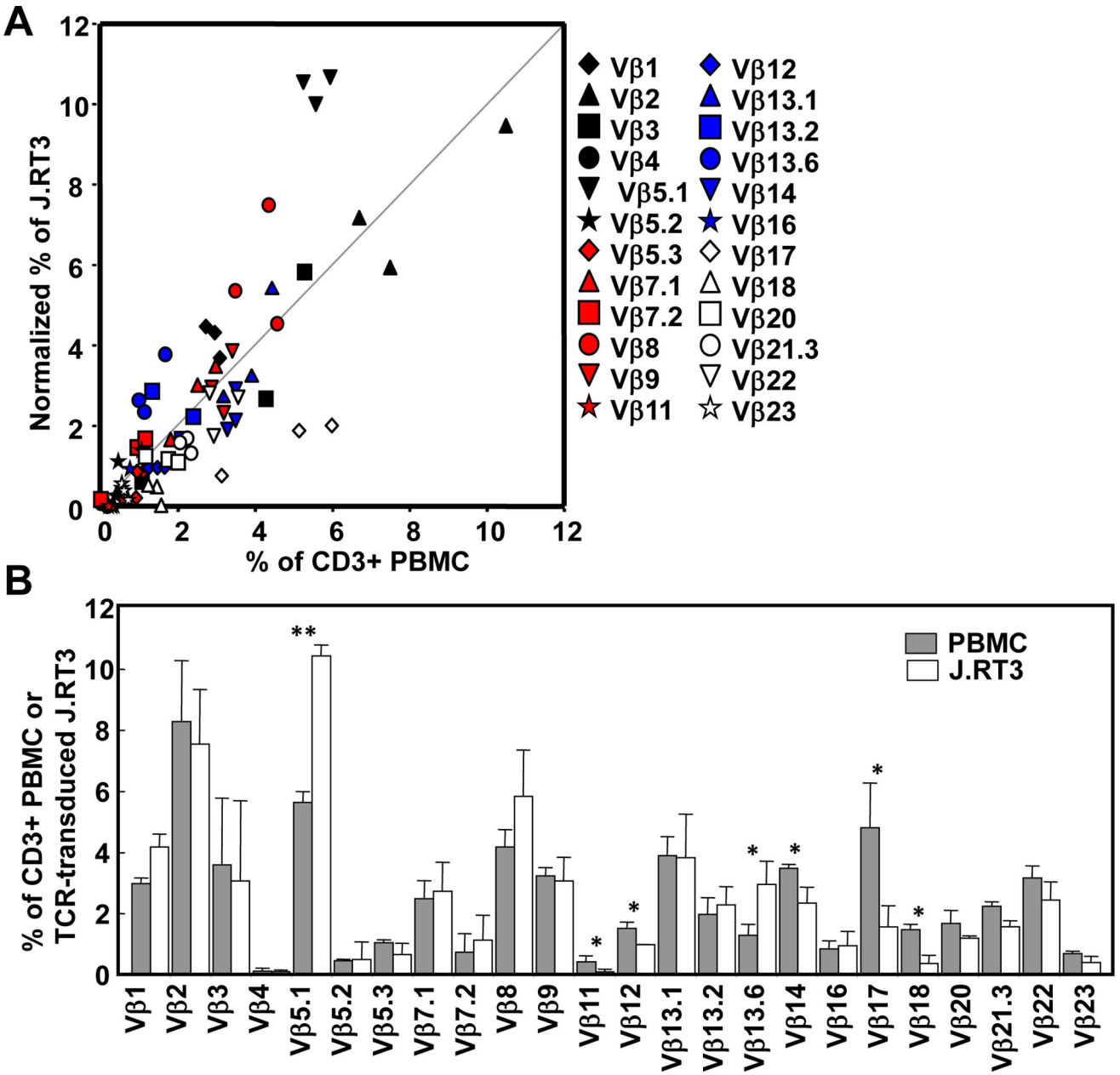

Figure 3.

Construction and characterization of TCR-gene library of peripheral polyclonal T cells. Vβ subtype frequencies in peripheral T cells and TCR gene library-transduced J.RT3 were compared. Frequencies of Vβ-subtype expressing $CD3^+$ T cells in PBMC and TCR gene library-transduced J.RT3 were determined by flow cytometry. Because of the limited availability of Vβ subtype-specific antibodies, total percentages of peripheral T cells that were stained by any of these antibodies were 59, 62, and 57% for 3 donors. Frequency for J.RT3 was normalized to the corresponding percentages in peripheral T cells. **A,** Frequencies of Vβ-subtype expressing $CD3^+$ PBMC were plotted against the corresponding values in J.RT3. **B,** Mean frequencies for $CD3^+$ PBMC and J.RT3 were compared. Each bar shows mean frequency and the standard deviation. Statistical significance is shown as * ($P < 0.05$)

and ** ($P < 0.01$) by paired t test. Each experiment was performed at least twice with similar results.

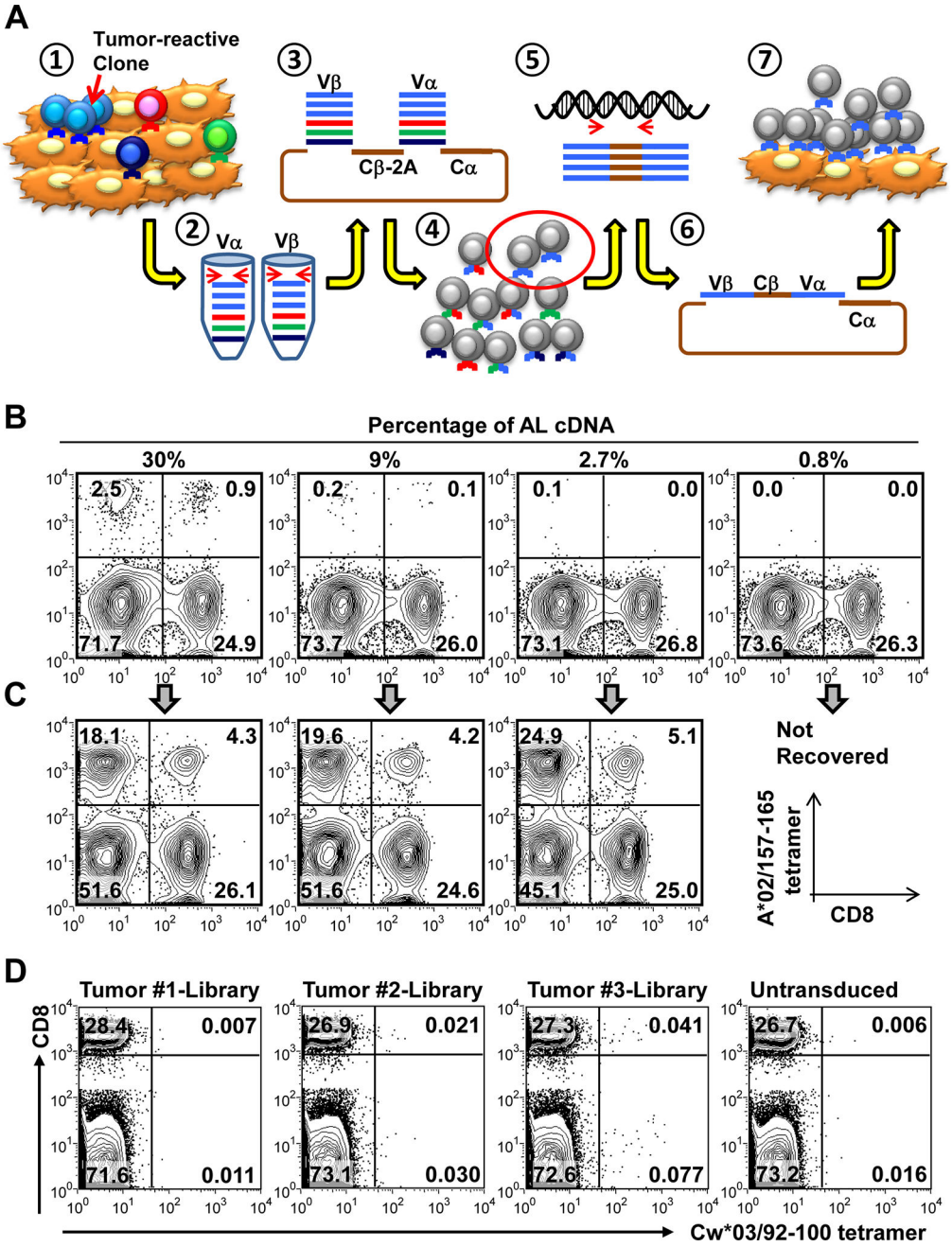

Figure 4.

Construction of tumor-derived TCR gene library. **A,** Procedures for TCR gene library construction and screening. (1) Tumor specimens that are enriched with tumor-reactive T cells were selected for experiments. (2) TCR genes were amplified from cDNA and (3) randomly assembled into the destination plasmid vector together with the β-chain constant region-P2A fusion gene fragment. (4) Activated peripheral T cells from healthy individuals were transduced with the TCR library and sorted for tumor antigen-specificity using the MHC/peptide tetramer reagent. (5) TCR transgenes were amplified from genomic DNA of the sorted tetramer-stained cells and (6) re-assembled into the destination vector. (7)

Activated T cells were transduced by the secondary library and tested for tumor antigen specificity. **B**, Randomly paired TCR-expressing libraries were constructed from mixtures of normalized cDNA of HLA-A*02-restricted NY-ESO-1(157–165)-specific AL and irrelevant T-cell clones. Polyclonally activated T cells were transduced by retroviral libraries and stained by A*02/NY-ESO-1(157–165) tetramer. **C**, Tetramer^{+} T cells in **B** were sorted and integrated TCR-expressing transgenes were amplified and re-assembled in TCR-expressing plasmid to create the secondary library. Polyclonally activated T cells were transduced by the secondary libraries and stained by A*02/NY-ESO-1(157–165) tetramer. **D**, Staining of T cells transduced with the tumor-derived TCR library by HLA-Cw*03/NY-ESO-1(92–100) tetramer. Activated T cells were transduced with the tumor-derived TCR-expression retroviral library. Two days after transduction, cells were stained by the tetramer followed by CD8. Each experiment was performed at least twice with similar results.

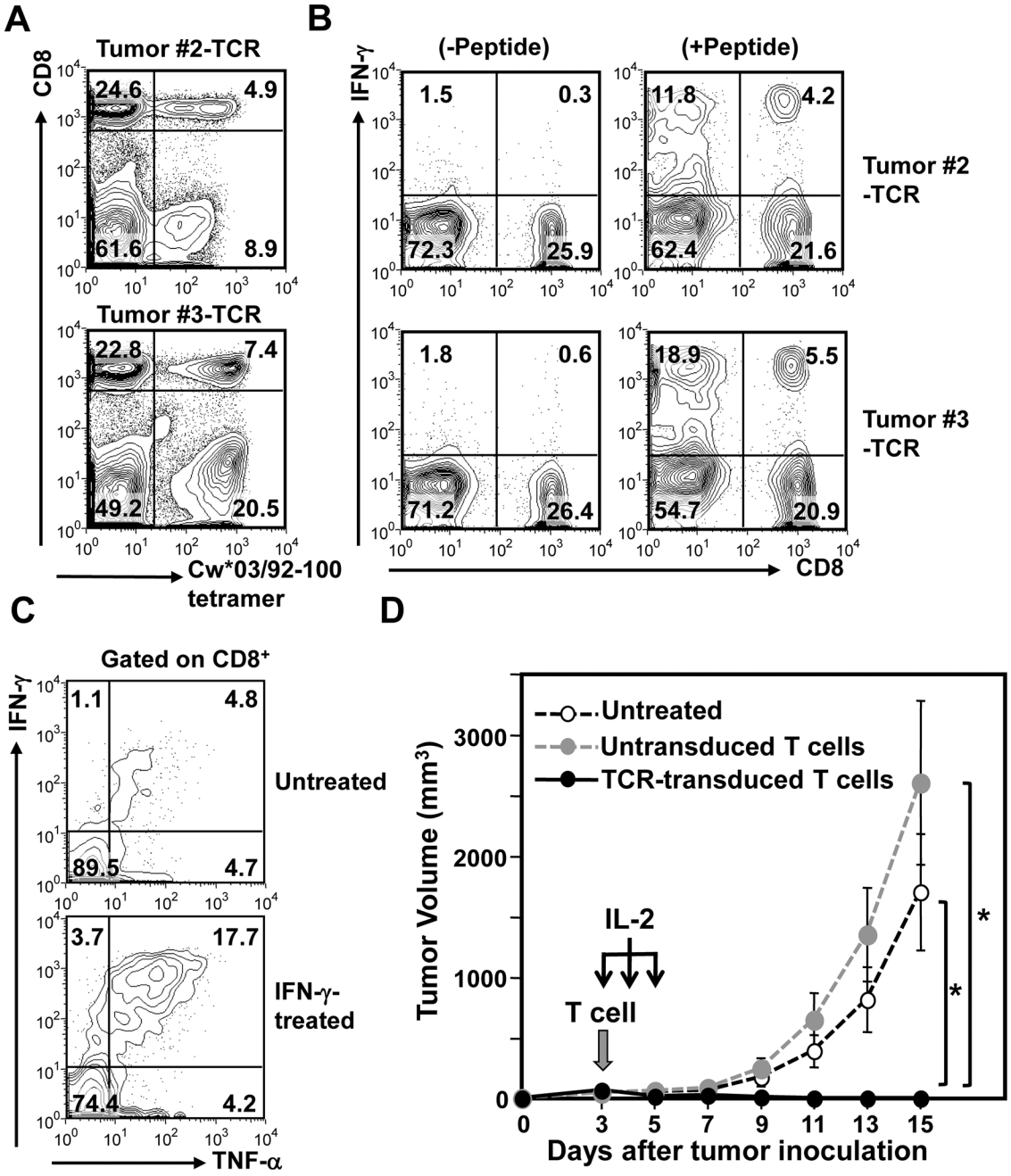

Figure 5.

Characterization of library-derived tumor antigen-specific TCR. **A**, Tetramer staining of T cells transduced with the secondary TCR gene library. **B,** Reactivity of the secondary TCR gene library-transduced T cells against the cognate peptide. Transduced T cells were cocultured with NY-ESO-1(92–100) peptide-pulsed or unpulsed Cw*03^{+} target cells for 6 hours in the presence of Monensin and intracellularly stained for IFNγ following cell-surface CD8 staining. **C,** Reactivity to cancer cells. Cw*03^{+}NY-ESO-1^{+} A2780 were treated with or without IFNγ for 2 days and were used as target cells in intracellular IFNγ staining of tumor #3-derived bulk secondary TCR library-transduced T cells. **D,** Therapeutic effect of

secondary TCR gene library-transduced T cells. Cw*03^{+}NY-ESO-1^{+} A2780 were treated *in vitro* with IFNγ for 2 days and subcutaneously inoculated into NSG mice. On day 3, mice were infused with the tumor #3-derived bulk secondary TCR library-transduced or untransduced T cells. Controls were left untreated. Tumor growth was monitored by measuring tumor diameters. Statistical significance ($P < 0.05$) is shown as * by *t* test. Each experiment was performed at least twice with similar results.