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Research paper

Improving TCR affinity on 293T cells

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ABSTRACT

This study presents an efficient method to improve TCR affinity, comprising 1) CDR-directed saturation mutation of TCR cDNA, 2) transient TCR display on CD3-expressing HEK293T (CD3-293T) cells by simple plasmid transfection, 3) staining with HLA-tetramers, and 4) multi-round sorting of cells with CD8-independent tetramer binding on a flow cytometer. Using these procedures, we successfully identified mutant TCRs with enhanced binding from an HLA-A*24:02-restricted, human telomerase reverse transcriptase (hTERT)-specific TCR. Two such clones, 2A7A and 2D162, harboring mutations in CDR1 and CDR2 of TCRβ, respectively, were isolated with both showing sequential four amino acid substitutions. When expressed on CD3-293T cells along with wild-type TCRα, the TCR molecules of these mutants as well as their combinatory mutation, bound to HLA-A24/hTERT-tetramers more strongly than the wild-type TCRs, without binding to control tetramers.

Besides, in order to facilitate a functional study of TCR, we established an artificial T cell line, designated as CD8I-J2, which expresses a human CD8 and IFN-γ producing cassette by modifying Jurkat-derived J.RT3-T3.5 cells. CD8I-J2 cells expressing wild-type or affinity-enhanced hTERT-specific TCRs were analyzed for their recognition of serially diluted cognate peptide on HLA-A*24:02-transduced T2 cells. CD8I-J2 cells expressing each mutant TCR recognized the hTERT peptide at lower concentrations than wild-type TCR. The hierarchy of peptide recognition is concordant with tetramer binding on CD3-293T cells and none of these mutant TCRs were cross-reactive with irrelevant peptides reported to be present on HLA-A*24:02 molecules as far as tested. These methods might thus be useful for obtaining high affinity mutants from other TCRs of interest.

1. Introduction

Cancer cells display endogenous peptides bound to major histocompatibility complex (MHC) molecules on their surface. $CD8^+$ cytotoxic T lymphocytes (CTLs) survey peptides through cognate recognition by T cell antigen receptors (TCRs) and destroy the target cells. Accordingly, CTLs reactive to tumor-associated antigens play important roles in cancer immunity, and several targets are non-mutated self-proteins because cancers often selectively express or over-express these as tumor-associated antigens (TAAs). For example, cancer-testis antigens such as NY-ESO-1 and MAGE proteins are selectively expressed in some cancers and in the testes that usually lack HLA expression (Simpson et al., 2005). Therefore, such protein types are considered ideal target antigens for CTL-based immunotherapy (Kawakami et al., 1994). In other cases, target proteins are substantially expressed in both cancer and normal tissues. Indeed, most melanoma-associated antigens such as MART-1, gp100, and tyrosinase, are also found in normal melanocytes of the skin, eye, and ear. Additionally, these normal organs are susceptible to the adoptive transfer of highly active MART-1-, or gp100-specific TCR-transduced T cells (Johnson et al., 2009).

Human telomerase complexes are composed of a telomerase RNA component, telomerase protein 1, and human telomerase reverse transcriptase (hTERT) (Nakayama et al., 1997; Harrington et al., 1997). Messenger RNA expression of hTERT is essential for telomerase activation during cellular immortalization and tumor progression, and has been frequently demonstrated in telomerase-positive primary tumors and cancer cell lines; however, it was found to be low or undetectable in normal tissues (Meyerson et al., 1997; Bodnar et al., 1998). Thus,

Abbreviations: CD3-293T, CD3 expressing HEK-293T; CD8I-J2, CD8 expressing and IFN-γ producing Jurkat-derived J.RT3-T3.5; hTERT, human telomerase reverse transcriptase; T2-A24, HLA-A*24:02-transduced T2

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hTERT could be a candidate universal tumor antigen for TCR-based immunotherapy (Ugel et al., 2010; Okamoto et al., 2012; Miyazaki et al., 2013).

Adoptive transfer of T lymphocytes with engineered specificity for tumor antigens is a promising approach to target cancer (Restifo et al., 2012). Recent and emerging clinical data have revealed potent anti-tumor activity in patients receiving such treatment (Morgan et al., 2006; Johnson et al., 2009; Chodon et al., 2014). However, because most tumor antigens are derived from self-proteins, isolation of high-affinity tumor-specific T cells is effectively precluded by thymic selection. Wherever such T cells are isolated, their TCRs typically have a weaker affinity for peptide–MHC complexes compared to virus-specific counterparts (Aleksic et al., 2012). TCR affinity can be modulated by mutation of specific residues within the complementarity-determining regions (CDRs) (Chlewicki et al., 2005; Li et al., 2005; Dunn et al., 2006) to generate TCR complexes with enhanced affinity for specific peptide-MHC complexes. Substitution of a few amino acids within CDRs can substantially enhance the affinity of TCRs to recognize target antigens (Robbins et al., 2008; Schmid et al., 2010). Considerable increases in TCR antigen affinity have been reported and development of engineered, affinity-enhanced TCRs is emerging as a powerful strategy to effectively target tumors and expand the opportunities for TCR-based adoptive T cell therapies (Varela-Rohena et al., 2008; Robbins et al., 2011; Rapoport et al., 2015).

We have previously attempted to enhance the affinity of TCR isolated from an HLA-A24-restricted hTERT-specific CTL clone originating from a healthy adult donor (Tajima et al., 2004a). Although phage display is an effective method for some TCRs, we could not obtain such TCR variants using this method. Alternatively, we established a TCR display system using HEK293T cells. Combined with flow cytometry-mediated cell sorting, we could successfully isolate affinity-enhanced TCRs in the present method.

2. Materials and methods

2.1. cDNA isolation and plasmid construction

cDNAs encoding the TCRα or TCRβ chain were isolated from an HLA-A*24:02-restricted hTERT-specific CTL clone, K3–1 (Tajima, 2004a), according to previously described methods (Kobayashi, 2013), and independently inserted into a pcDNA3.1 vector (Life Technologies, CA, USA). The Vα gene allele was TRAV29/DV5*01, TRAJ34*01 and the Vβ gene allele was TRBV20-1*02, TRBJ2-1*01, TRBD2*01.

The cDNA of four CD3 subunits was isolated from T cells by RT-PCR, linked with self-cleaving porcine 2A peptide (Kim et al., 2011) using overlapping PCR and In-Fusion (Takara Bio, USA, Inc., Mountain View, CA) techniques, and inserted into an episomal vector, pEBMulti-Bsd (Wako Chemicals, Osaka, Japan). Human CD8α cDNAs were also isolated and inserted into the CSII-EF-MCS plasmid (a kind gift from Dr. Miyoshi, Department of Physiology, Keio University School of Medicine). All plasmids were verified by DNA sequencing. This study was approved by the institutional review board of the Aichi Cancer Center.

2.2. Construction of TCR library containing CDR mutations

Amino acid degenerate codon libraries were created by site-saturated mutagenesis using overlapping extension PCR as described previously (Williams et al., 2014). For hTERT TCR, five or four amino acid degenerate codon libraries, overlapping by several amino acids for each CDR of α- and β- chains were constructed as described above.

2.3. Production of HLA-monomers and tetramers

HLA/peptide monomers and tetramers were produced as described previously (Kuzushima et al., 2001). Briefly, recombinant HLA-A*02:01 or A*24:02 protein, β_2m, and CTL epitope peptides were incubated in a refolding buffer. Properly refolded HLA molecules were purified by FPLC and biotinylated using biotin-protein ligase (Avidity, LLC, Aurora, CO, USA). After second purification by FPLC, HLA monomers were incubated with PE-labelled streptavidin for tetramer production. HTLV-1 $tax_{11–19}$ (LLFGYPVYV) and EBV $LMP1_{125–133}$ (YLLEMLWRL) peptides were incorporated into HLA-A2 molecules. The peptides incorporated into HLA-A24-molecules included $hTERT_{461–469}$ (VYGFVRACL) and HIV-1 $envelop_{584–592}$ (RYLRDQQLL). All peptides were synthesized by Toray Research Center (Kamakura, Japan).

2.4. Establishment of human CD3-expressing 293T cells

The pEBMulti-Bsd vector tandemly expressing four CD3 subunits was transfected into HEK-293T cells using Lipofectamine 2000 (Invitrogen). Two days after transfection, Blasticidin S (Wako) was added to a concentration of 7 μg/ml. The cells were further cultured in D-MEM (Sigma) containing 10% FCS, penicillin, streptomycin, GlutaMAX (Gibco) and Blasticidin S until used for transfection.

2.5. TCR display on 293T cells and selection of high affinity clones

TCR display on CD3-293T cells and selection of high affinity clones was performed as described previously with modifications (Ho and Pastan, 2009). Briefly, each TCR library plasmid (500 ng) was transiently transfected into human CD3-293T cells (6×10^5) along with the wild-type TCR partner plasmid (500 ng) in the well of a 12-well plate. After two to four days, cells were harvested, stained with PE-labelled HLA-tetramers at a final concentration of 10 μg/ml for 15 min at room temperature. FITC-labelled anti-CD3 antibody (Beckman Coulter) was then added and cells were incubated for an additional 15 min on ice. After washing twice with PBS, the cells were analyzed on a FACSAriaIII system (BD Biosciences) and the data were analyzed using FlowJo software (FlowJo, LCC). Cells with a stronger PE signal than those transfected with wild-type TCR pairs specific to hTERT were sorted simultaneously. A mixture of α- and β-TCR plasmids was recovered from the selected cells using QIAprep Spin Miniprep Kit (Qiagen GmbH, Hilden, Germany) and transformed into NEB10-beta *E. coli* competent cells (New England Biolabs Inc., Ipswich, MA) for further use including DNA sequencing.

2.6. Tetramer binding assay

The pcDNA3.1 plasmids harboring wild-type hTERT-TCRβ, a TCRβ CDR1-mutant (2A7A), a TCRβ CDR2-mutant (2D162), and their combination (2A7A-2D162) were individually transfected into CD3-293T cells along with the pcDNA3.1/hTERT-TCRα plasmid. After three days, cells were stained with PE-labelled HLA-A24-tetramers incorporating the cognate hTERT peptide or an irrelevant HIV peptide at various concentrations. In some experiments, cells were stained with anti-TCRαβ monoclonal antibody recognizing a conformational epitope (clone WT31, BD Biosciences). After washing, the cells were analyzed on a FACSAriaIII system and the data were analyzed using FlowJo software.

2.7. Artificial T cells expressing wild-type and affinity-enhanced hTERT-specific TCR and producing IFN-γ in response to TCR stimulation

J.RT3-T3.5 (hereafter referred as J2) cells, a TCRβ-deficient Jurkat cell derivative was purchased from the ATCC (Summit Pharmaceuticals International Corporation, Tokyo, Japan). CD8α cDNA was lentivirally transduced into these cells as described previously (Demachi-Okamura et al., 2012), and a stable CD8α expressing clone, designated as CD8-J2, was obtained from limiting dilution cultures. To harness the capability of producing IFN-γ upon TCR ligation to the CD8-J2 cells, four repeats of NFTA binding elements were tandemly linked to the minimal β-globin promoter, and inserted into a pRetroX-Tight-Pur vector

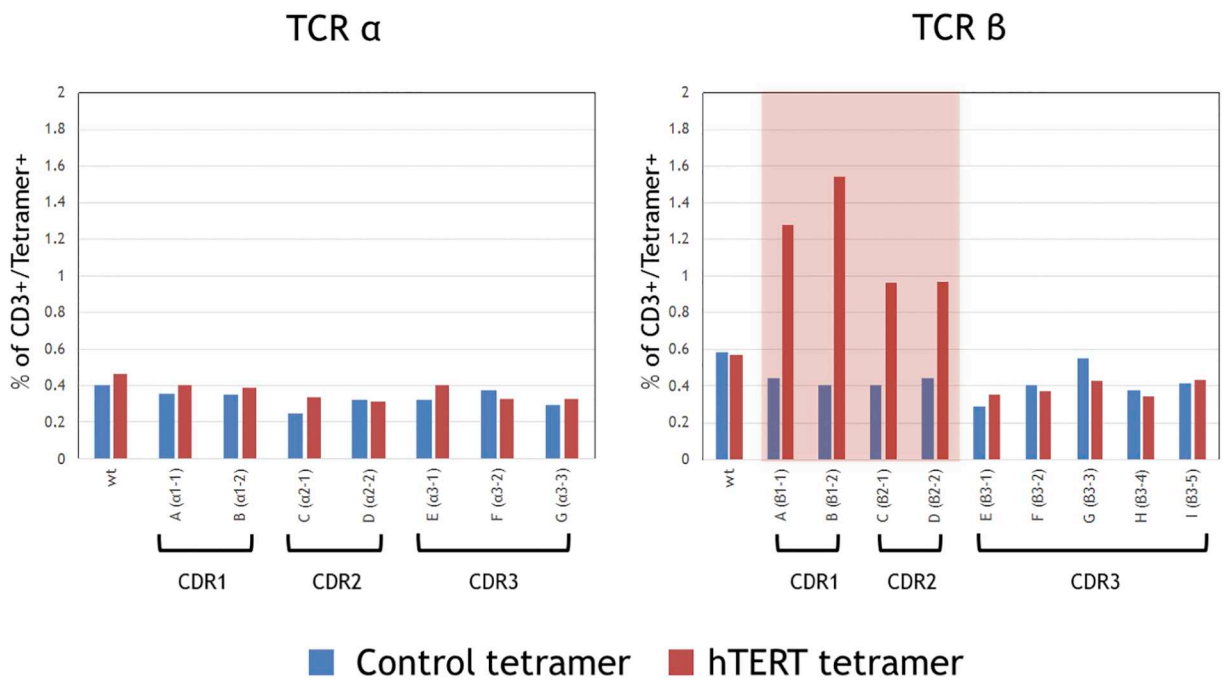

Fig. 1. TCR display and screening of pcDNA3.1 plasmid libraries. Each CDR library with random mutation at 5 amino acids (except for the CDR3-5 library of TCRβ, having a 4 amino acid mutation), was transfected into CD3-expressing HEK293T cells along with its wild-type partner TCR. The percentage of CD3 + /tetramer + cells is shown. Cells with enhanced tetramer binding are indicated in the red shaded area. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Clontech Laboratories, Inc. Mountain View, CA), from which Tet-responsive promoter was deleted in advance. CD8-J2 cells were infected with the resultant retrovirus, cultured in the presence of puromycin for selection, and designated as CD8I-J2 cells.

Porcine 2A peptide was used to link β- and α- TCR chains and the resultant fusion genes were inserted into the LZRSpBMN-Z vector (a kind gift from G. Nolan, Stanford University, Stanford, CA) after removing the lacZ gene as described previously (Kondo et al., 2002). These vectors were transduced into Phoenix-GP cells (ATCC) expressing the Gibbon ape leukemia virus envelope isolated from PG-13 (ATCC) to produce retrovirus in the supernatant. CD8-IFN-J2 cells were infected with the retrovirus, cultured, and stained with PE-labelled HLA-A24/hTERT-tetramers. Tetramer-positive subpopulations were isolated using anti-PE MACS beads and magnetic columns (Miltenyi) according to the manufacturer's instructions. These cells were used as effector cells in the peptide recognition assay.

2.8. Peptide recognition assay

T cell responses to synthetic peptides were analyzed as described previously (Kondo et al., 2013). Briefly, transporters associated with antigen processing-deficient T2 cells transduced with HLA-A*24:02 (T2-A24), were pulsed with serial concentrations of the hTERT peptide or a fixed concentration (1 μM) of irrelevant peptides reported to be present on the HLA-A*24:02 molecules (Supplemental Table 1). TCR-transduced Jurkat cells (10,000) were added to each well. On the following day, culture supernatants were subjected to IFN-γ ELISA.

2.9. Enzyme-linked immunosorbent assay (ELISA)

Supernatants were harvested and analyzed for IFN-γ concentrations using ELISA. Briefly, ELISA plates (Corning, NY, USA) were pre-coated with the anti-human IFN-γ monoclonal antibody (M700A; Thermo SCIENTIFIC, MA, USA) at 4 °C overnight. After blocking, the supernatants were incubated for 1 h at room temperature. The plates were subsequently incubated with a biotin-labelled IFN-γ monoclonal antibody (M701B; Thermo SCIENTIFIC, MA, USA), followed by the addition of horseradish peroxidase-conjugated streptavidin and incubation for 30 min at room temperature. For colour visualization, tetramethylbenzidine substrate (Sigma-Aldrich) was added, and absorbance was measured at 630 nm. Between each step, the plates were washed six times with PBS containing 0.05% Tween-20.

3. Results

3.1. Failure of affinity improvement by phage display and establishment of a TCR display system using 293T cells

In an attempt to obtain TCR with higher affinity, we carried out phage display as reported previously (Li et al., 2005). However, we could not detect positive results with our HLA-A24-restricted hTERT-specific TCR, and affinity enhancement and clonal concentration were observed in our setting when we used HLA-A2-restricted HTLV1 tax-specific-TCR, A6 (Supplemental Fig.1) as reported previously (Li et al., 2005). We thus suspected that hTERT TCR might not be expressed on the phage surface as abundantly as A6.

We thus aimed to establish a novel TCR expression and selection system using HEK293T cells, based on a report describing the successful selection of high affinity scFV antibodies on these cells (Ho and Pastan, 2009). To this end, we first introduced human CD3 subunits, γ, δ, ε, and ζ, linked by porcine 2 A peptides into 293T cells and obtained a stable cell line, designated as CD3-293T, after Blasticidin S selection. Upon transfection with α- and β- hTERT-TCRs, the CD3-293T cells showed low expression of the hTERT TCR heterodimers, which were only marginally stained with HLA-A24/hTERT-tetramers. This poor staining may result from the absence of co-receptor CD8 molecules on these cells. We exploited this situation, which enabled us to isolate mutant TCRs that could bind in a CD8-independent manner.

Secondly, we constructed a total of 16 TCR libraries randomly mutated at 4 or 5 amino acids in each of the α- or β- CDR, with some amino acids overlapped by adjacent libraries (Supplemental Table 2). We included CDR1 and 2 as mutation targets, because some of the

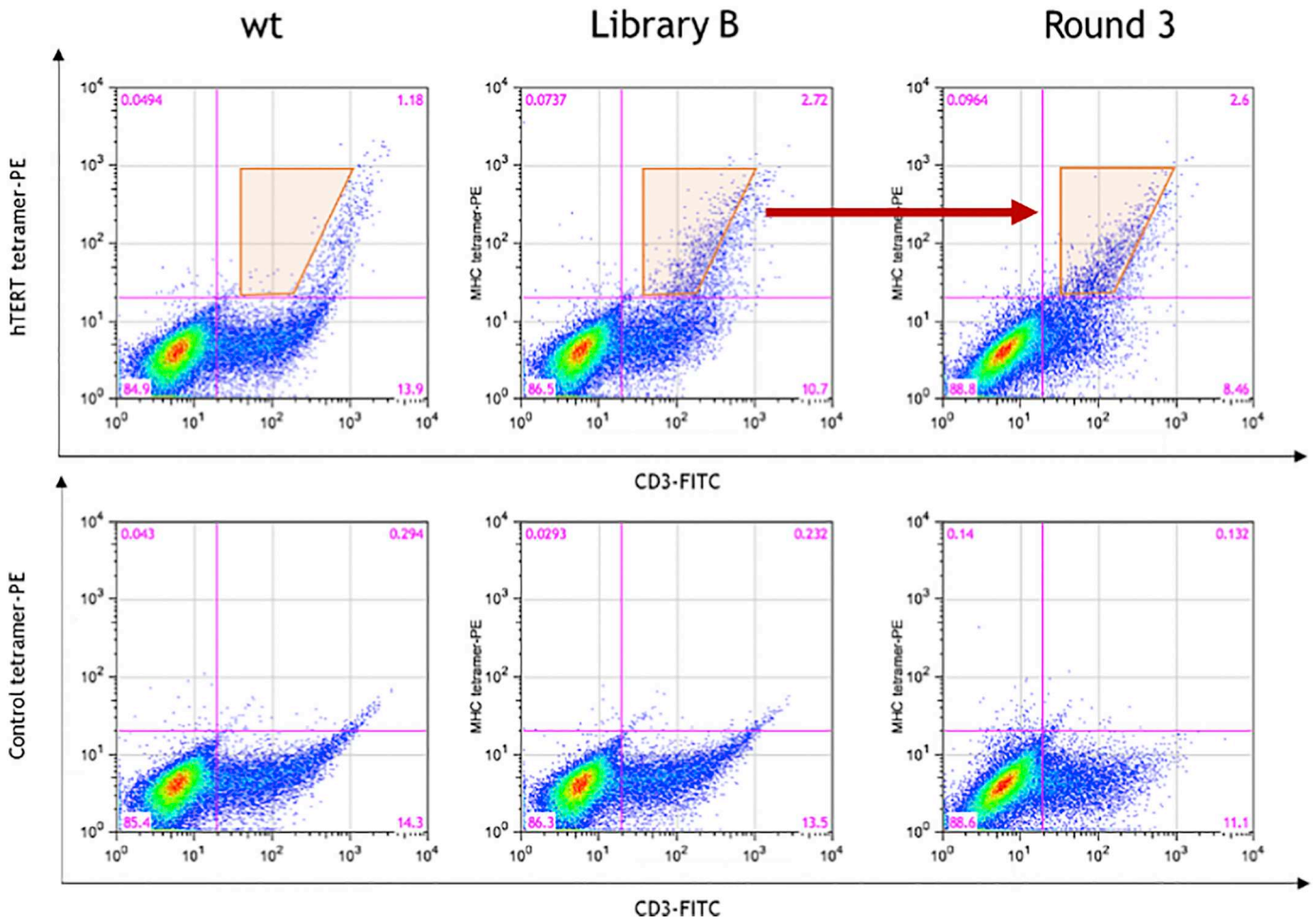

Fig. 2. Cell sorting on a flow cytometer. CD3-293T cells were transfected with either wild-type (wt) hTERT TCRβ or the library B plasmid, corresponding to TCRβ CDR1-2, harboring a saturated mutation in TCRβ CDR1, along with wild-type TCRα. Each transfected cell group was stained with a CD3 antibody and HLA-A24 tetramers incorporating the hTERT or HIV env peptide (as a control). Cells with brighter tetramer signals (red shadowed area) were sorted. Plasmids were recovered from the sorted cells and transfected into CD3-293T cells for the next flow cytometric analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mutations were reported to contribute to affinity augmentation while retaining the TCR specificities (Li et al., 2005; Dunn et al., 2006). Each plasmid library was transfected into CD3-293T cells along with its wild-type partner TCR. Tetramer staining results for the transfected cells are demonstrated in Fig. 1. Cells with enhanced tetramer binding were observed in libraries with mutations at CDR1 and CDR2 of TCRβ. These results indicate that substitution of amino acids in these regions can enhance TCR affinity.

3.2. Sorting of affinity enhanced TCR expressing cells and characterization of the mutation

Next, we collected cells with brighter signals of HLA-A24/hTERT-tetramers on the cell sorter. Plasmids were recovered from the sorted cells, and transfected into CD3-293T cells. As exemplified in Fig. 2, After 2 rounds of selection, cell populations with stronger signals were increased in all the four TCRβ libraries (CDR1-1, CDR1-2, CDR2-1, CDR2-2). After 3 rounds of selection, there was no further increase. Thus, we picked up individual bacterial colonies from the round 2 selection and purified each plasmid.

When individual mutant TCRβ plasmids were transfected into CD3-293T cells along with wild-type TCRα, some cells expressed TCRs which bound to HLA-A24/hTERT-tetramers stronger than did wild-type TCRs (Fig.3). These mutant TCRs did not bind to negative control HLA-A24/HIV env-tetramers. The amino acid sequences of some of the CDR1 and CDR2 mutations are listed in Fig. 3. The mutation of glutamine (Q) to proline (P) at position 29 and threonine (T) to arginine (R) at position 31 in CDR1 appears to confer affinity enhancement. Mutation of asparagine (N) to basic amino acids such as lysine (K) or R at position 51 and lysine (K) to glycine (G) at position 55, in CDR2 appears to be important for stronger binding.

To gain insight into the amino acid contribution to higher receptor binding in clones 2A7, 2C40, and 2D162, we constructed plasmids with a reduced number of amino acid mutations. As demonstrated in Fig. 4, reversal of R to T to at position 31 in clone 2A7 resulted in almost abolished binding, indicating the important role of R at this position. Reversal of P to Q at position 29 substantially reduced the binding. These findings are in concordance with the preferential usage of these amino acids in high binders found among CDR1 mutants as shown in Fig. 3. Interestingly, only one or two amino acid(s) mutation, such as R at position 31, or P and R at position 29 and 31, respectively yielded significant improvement of binding, again underscoring the critical roles of these amino acids. Simultaneously, we identified clone 2A7A as the best binder among 2A7 derivatives.

Among four amino acids mutated in clone 2C40, K and phenylalanines (F) at position 51, 52, and 53, respectively, had similar contribution for improved binding, whereas N at position 54 had none. Concerning the four amino acids mutated in clone 2D162, each revertant showed an apparently decreased binding, indicating the substantial contribution of all amino acids.

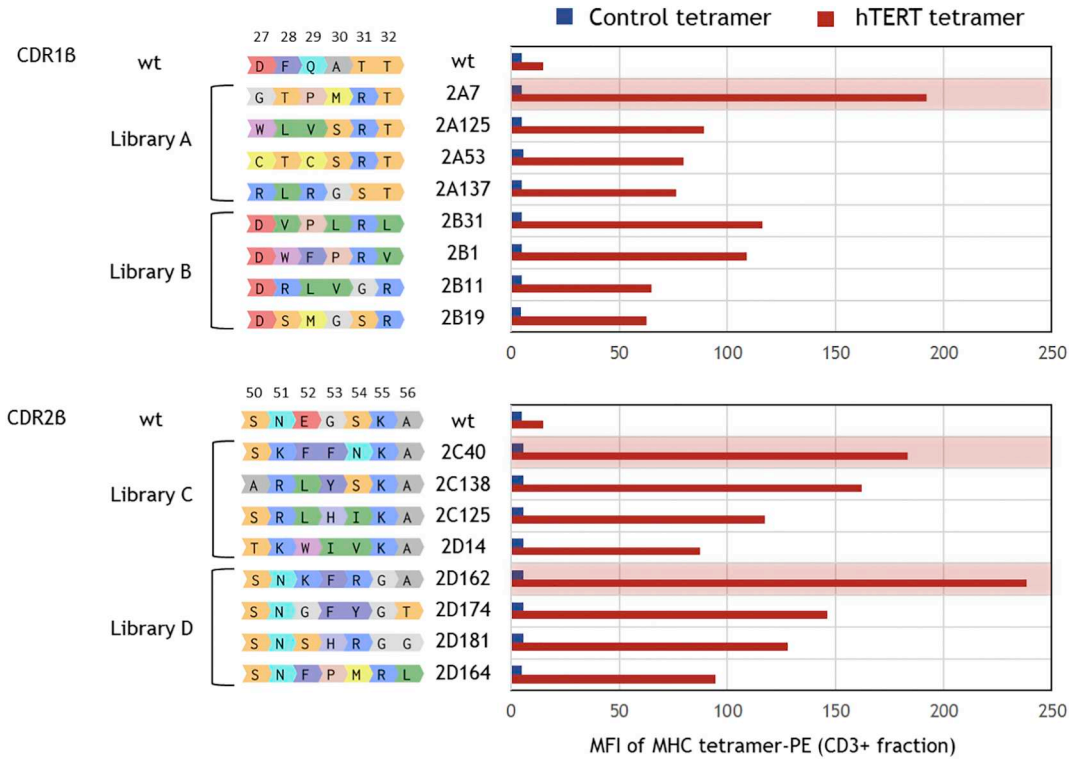

Fig. 3. Analysis of individual mutant TCRβ plasmids isolated by multiple rounds of cell sorting. Each mutant plasmid was transfected into CD3-293T cells along with wild-type TCRα. After 3 days of incubation, cells were stained with anti-CD3 antibody and HLA-A24/hTERT-tetramer or HLA-A24/HIV env-tetramer as a negative control. Amino acid sequences of CDR1 and CDR2 mutations are shown using the one letter code. Clones with red-shadowed bars (2A7, 2C40 and 2D162) were subjected to further analysis. The mean fluorescence intensity (MFI) of CD3+/tetramer+ fractions is shown.

3.3. Evaluation of representative CDR1 and CDR2 mutants in biochemical and functional studies

We selected clone 2A7A and 2D162 as the best binders from CDR1 and CDR2 mutants of TCRβ, respectively. Both clones contain sequential four amino acid substitutions. We also constructed their combinatory mutation, designated as 2A7A-2D162. These three plasmids were transfected into CD3-293T cells with wild-type TCRα. After three days, the cells were stained with HLA-A24-tetramers incorporating hTERT or HIV peptide. As demonstrated in Fig. 5, the combination of 2A7A and 2D162 mutations resulted in an additive effect for binding without compromising the peptide-specificity.

To exclude the possibility that augmented tetramer binding merely reflects improved TCR protein expression caused by CDR mutation, wild-type and mutant hTERT TCRβ plasmids were transfected along with wild-type TCRα into CD3-293T and the cells were stained with anti-TCRαβ antibody recognizing a conformational epitope or HLA-A24/hTERT-tetramers. As demonstrated in Supplemental Fig. 2A, tetramer binding increased without improvement of TCR expression on the cell surface. Percent and mean fluorescence intensity of CD3+/TCRαβ+ fractions is not significantly different among cells transfected with wild-type and the mutant plasmids at various infected doses (Supplemental Fig. 2B and C). These results indicate that augmented tetramer binding is not result of increase of TCR protein expression.

Finally, we established CD8I-J2 cells expressing the wild-type or affinity-enhanced hTERT-specific TCRs to study the TCRs functionally. Supplemental Fig. 3 demonstrates the results of tetramer staining in these cells. These cell lines were analyzed for their recognition of a serially diluted cognate peptide on T2-A24 cells. As demonstrated in (Fig. 6), CD8I-J2 cells expressing mutant TCRs recognized the hTERT peptide at lower concentrations than did wild-type TCRs. The hierarchy of peptide recognition is concordant with tetramer binding as shown in Fig. 5. None of these TCRs were cross-reactive with irrelevant peptides reported to be present on the HLA-A*24:02 molecules (supplemental Fig. 4. and Table 1) as far as tested.

4. Discussion

Several methods for TCR display are available to enhance TCR affinity to cognate peptides on restricted MHC molecules. One frequently reported method is the phage display technique; however, we assume that there might be hidden difficulties or tricks behind these protocols, especially for certain TCRs, because only a few groups could successfully isolate such T cells using this technique. Indeed, we failed to isolate high affinity variants from our hTERT-specific TCR using phage display, but could successfully repeat the A6 TCR affinity maturation as demonstrated previously (Li et al., 2005). Other methods for TCR display include yeast display; however, this method relies on a single-chain format of TCR molecules and is applicable only to the Vα2 (IMGT:TRAV12-2) family of human TCRs so far. Several reports have described mammalian display methods of engineering a combinatorial library of TCR mutants on the surface of TCR-negative T cells (Kessels et al., 2000; Chervin et al., 2008; Malecek et al., 2013). This strategy allows TCR expression on the T cell surface in complex with CD3 signaling subunits. However, one drawback of the mammalian T cell surface display is the limited potential for combinatorial library diversity (Richman and Kranz, 2007).

Epithelial cell lines are an alternative to tumor-derived T cell lines, which are often difficult for gene transduction, and show potent gene transduction. A few reports have described CD3-harboring HEK293T epithelial cells as potential candidates to display plasmids or short linearized TCR cDNAs (Walseng et al., 2015; Hamana et al., 2016). Further, a report describing the display of an scFV library on HEK293T cells and selection of high affinity clones by cell sorters (Ho and Pastan, 2009) encouraged us to establish a similar system for TCR.

We described here the details of directed evolution of HLA-24-

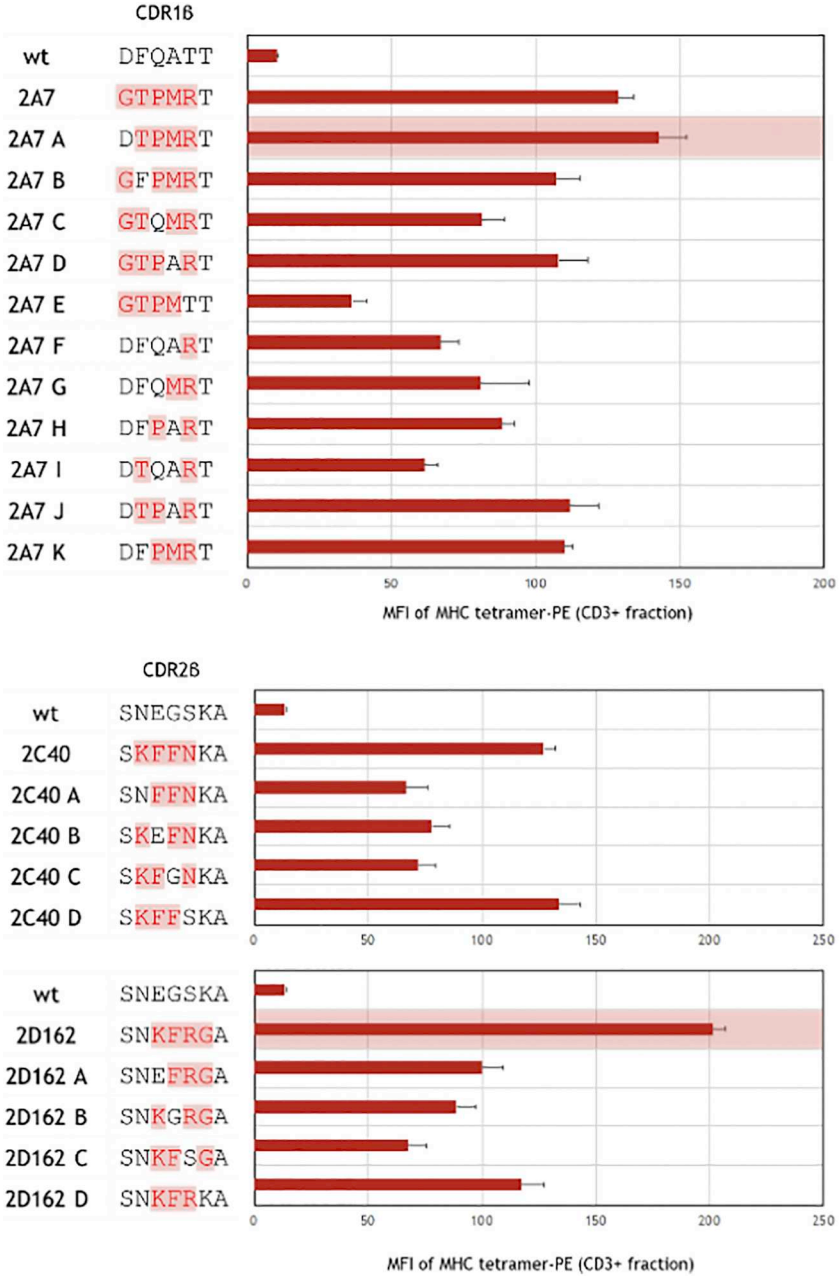

Fig. 4. Amino acid contribution to the binding of clone 2A7, 2C40, and 2D162 to the HLA-A24/hTERT-tetramer. Mutated amino acids are indicated in red colour. Clones with red-shadowed bars (2A7A and 2D162) were subjected to further analysis. The mean fluorescence intensity (MFI) of CD3 + /tetramer + fractions is shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

restricted hTERT-specific TCRs using CD3-expressing HEK293T (CD3-293T) cells. We constructed 7 libraries harboring saturated mutations at codons for 5 sequential amino acids covering CDR 1, 2, or 3 of TCRα and 9 libraries for TCRβ. There are several advantages of using CD3-293T cells in combination with the flow cytometer. First, they enable rapid screening of plasmid libraries in a row. As demonstrated in Fig. 1, we could efficiently determine the regions whose mutation could augment the hTERT-TCR affinity; for this TCR, in CDR1 and CDR2 of TCRβ. This was also true for other HLA-A24-restricted TCRs isolated from CTLs specific for CMVpp65 (Kuzushima et al., 2001) and Ep-CAM peptides (Tajima et al., 2004b) (Supplemental Fig. 5). Second, since the binding of TCRs on CD3-293T cells and HLA/peptide-tetramers is CD8-independent, chances of obtaining high affinity TCRs should be increased. Thirdly, high-end cell sorting technologies could enable efficient purification of mutated TCRs of interest. Lastly, episomal replication of the pcDNA3.1 plasmid containing the SV40 origin in CD3-293T cells expressing the large T-antigen, ensures that most of the plasmids can be recovered from sorted cells.

In parallel with demonstrating the clinical benefit of gene-engineered T-cell adoptive therapy, much concern has been recently raised on off-target toxicities of affinity-enhanced TCRs demonstrated as lethal adverse effects in individual patients (Linette et al., 2013; Cameron et al., 2013). Thus, TCRs with enhanced affinity should be examined through an array of *in vitro* assays to assess potential risks of on- and off-target toxicities (Ugel et al., 2010; Morgan et al., 2013; Kunert et al., 2017; Bijen et al., 2018) before clinical use.

5. Conclusion

We established a novel TCR display and selection technique for

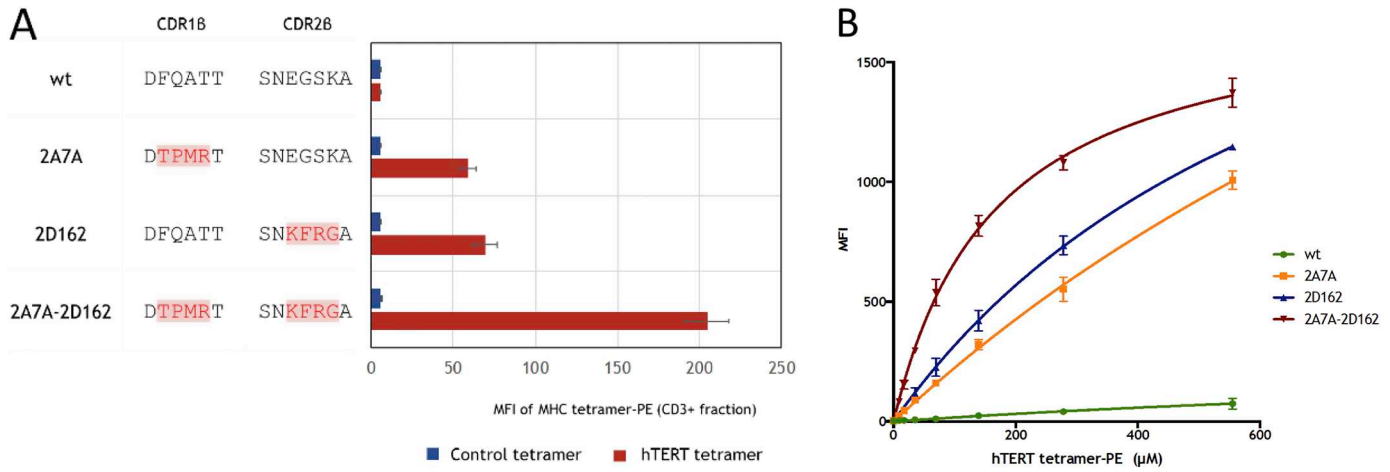

Fig. 5. Tetramer binding of wild-type and mutant hTERT TCRs. TCRβ plasmids harboring the wild type (wt), CDR1-mutant (2A7A), CDR2-mutant (2D162), and the combination (2A7A-2D162) were transfected into CD3-293T cells along with the TCRα plasmid. After three days, the cells were stained with PE-labelled HLA-A24-tetramers incorporating the cognate hTERT peptide or irrelevant HIV peptide at a fixed (A) and various (B) concentration(s). After washing, the cells were analyzed on a FACSAriaIII system and the data were analyzed using FlowJo software. The mean fluorescence intensity (MFI) of CD3 + /tetramer + fractions is shown.

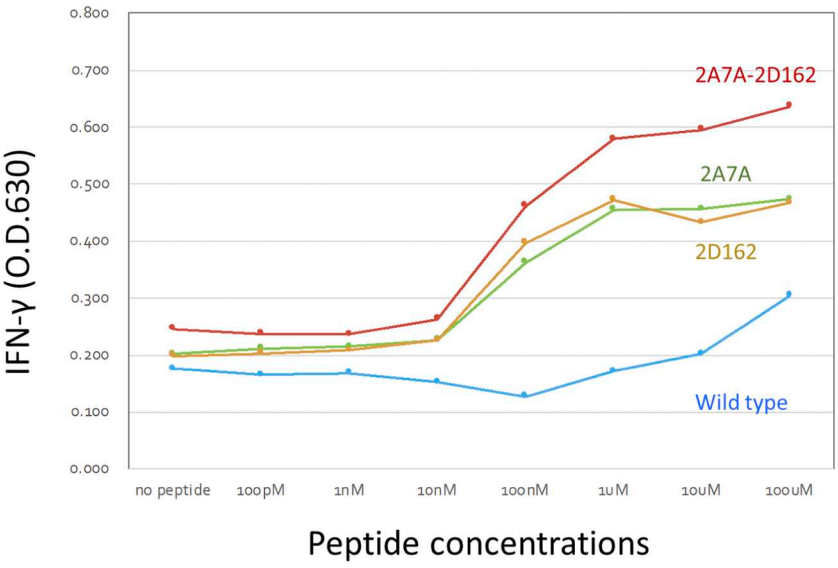

Fig. 6. T cells expressing mutant hTERT TCRs recognize lower concentrations of the cognate peptide than the wild-type TCR. T2-A24 cells were pulsed with serial concentrations of the hTERT peptide and incubated with TCR-transduced CD8I-J2 cells in wells of 96-well plates. On the following day, the culture supernatants were subjected to IFN-γ ELISA.

affinity selection. It comprises display on library-transfected 293T cells and sorting of cells of interest on a flow cytometer. Using the methods described here, we efficiently identified variant TCRs with enhanced cognate binding from an HLA-24-restricted hTERT-specific TCR. These methods might thus be useful for obtaining high affinity mutants from other TCRs as well.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2018.11.010.

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