Optimization of T-cell reactivity by exploiting TCR chain centricity for the purpose of safe and effective antitumor TCR gene therapy

Toshiki Ochi¹, Munehide Nakatsugawa¹, Kenji Chamoto¹, Shinya Tanaka¹,², Yuki Yamashita¹, Tingxi Guo¹,³, Hiroshi Fujiwara⁴, Masaki Yasukawa⁴, Marcus O. Butler¹,³,⁵, and Naoto Hirano¹,³

¹Immune Therapy Program, Campbell Family Institute for Breast Cancer Research, Campbell Family Cancer Research Institute, Princess Margaret Cancer Centre, University Health Network, Toronto, Ontario, Canada M5G 2M9; ²Takara Bio, Inc., Otsu, Shiga, Japan 520-2193; ³Department of Immunology, University of Toronto, Toronto, Ontario, Canada M5S 1A8; ⁴Department of Hematology, Clinical Immunology and Infectious Disease, Ehime University Graduate School of Medicine, Toon, Ehime, Japan 791-0295; ⁵Department of Medicine, University of Toronto, Toronto, Ontario, Canada M5S 1A8.

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Corresponding author

Naoto Hirano, MD, PhD

Princess Margaret Cancer Centre

610 University Avenue, Toronto, ON M5G 2M9, Canada

Phone: (416) 946-2190

Fax: (416) 946-6529

E-mail: naoto.hirano@utoronto.ca

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Abstract

Adoptive transfer of T cells redirected by a high affinity antitumor T-cell receptor (TCR) is a promising treatment modality for cancer patients. Safety and efficacy depend on the selection of a TCR that induces minimal toxicity and elicits sufficient antitumor reactivity. Many, if not all, TCRs possess cross-reactivity to unrelated MHC molecules in addition to reactivity to target self-MHC/peptide complexes. Some TCRs display chain centricity, in which recognition of MHC/peptide complexes is dominated by one of the TCR hemi-chains. In this study, we comprehensively studied how TCR chain centricity impacts reactivity to target self-MHC/peptide complexes and alloreactivity using the TCR, clone TAK1, which is specific for human leukocyte antigen-A*24:02/Wilms tumor 1235-243 (A24/WT1235) and cross-reactive with B*57:01 (B57). The TAK1β, but not the TAK1α, hemi-chain possessed chain centricity. When paired with multiple clonotypic TCRα counter-chains encoding TRAV12-2, 20, 36, or 38-2, the de novo TAK1β-containing TCRs showed enhanced, weakened, or absent reactivity to A24/WT1235 and/or to B57. T cells reconstituted with these TCRα genes along with TAK1β possessed a very broad range (>3 log orders) of functional and structural avidities. These results suggest that TCR chain centricity can be exploited to enhance desired antitumor TCR reactivity and eliminate unwanted TCR cross-reactivity. TCR reactivity to target MHC/peptide complexes and cross-reactivity to unrelated MHC molecules are not inextricably linked and are separable at the TCR sequence level. However, it is still mandatory to carefully monitor for possible harmful toxicities caused by adoptive transfer of T cells redirected by thymically-unselected TCRs.
Introduction

One challenge for the success of adoptive T-cell therapy is that thymic and/or peripheral tolerance impair our ability to prepare high avidity antitumor T cells from all cancer patients (1, 2). To overcome this issue, the concept of engineered T cells has been developed in which T cells are redirected to specifically recognize tumors by the transfer of genes encoding high affinity tumor-specific chimeric antigen receptors (CAR) or T-cell receptors (TCR) (3-7). These redirected T cells are endowed with specificity and strong reactivity for tumor cells by transducing high affinity antitumor CAR or TCR gene (3-7).

Selection of a well-characterized antitumor TCR is pivotal for TCR gene therapy to achieve clinically effective antitumor responses with minimal adverse events. Obviously, TCR-transduced T cells should possess functional avidity sufficient to recognize tumor cells. However, in order to achieve a favorable therapeutic window and avoid on-target off-tumor toxicity, the avidity should not be excessively high given the expression of target antigens in normal tissues (5, 7). In addition, the risk of off-target toxicity caused by cross-reactivity inherent to TCRs must be considered, since many, if not all, TCRs possess cross-reactivity to self and non-self MHC presenting other peptides (8-14). In recent clinical trials of TCR gene therapy, it was reported that cross-reactivity of an antitumor TCR to a self-HLA-presented different, non-tumor-derived peptide caused unwanted off-target adverse events in patients treated with redirected T cells (15, 16). Strategies to remove unwanted cross-reactivity of TCRs while preserving antitumor reactivity have yet to be established.

Accumulating evidence has demonstrated that antigen-specific T cells in the periphery harbor identical or near-identical sequences across unrelated individuals despite a potentially vast T-cell repertoire (17, 18). Interestingly, a subset of biased TCRα and β chains possess chain centricity and thereby can pair with multiple clonotypic counter-chains while preserving antigen specificity (19-22). Avidities for these TCRs that are composed of chain-centric hemi-chains appear to be diverse (20, 23, 24). T cells transduced with a dominant TCR hemi-chain demonstrate cross-reactivity to different
peptides presented by the same self-MHC molecules depending on the TCR counter-chain clonotypes while retaining their original antigen specificity (21). These observations suggest that affinity and cross-reactivity of a TCR comprising a dominant hemi-chain can be modulated by pairing it with a non-chain-centric counter-chain without perturbing antigen specificity.

Wilms tumor 1 (WT1) is a promising tumor-associated antigen, since WT1 expression is abundant in many cancers, but restricted to some types of normal tissue such as podocytes in the kidney, mesothelial cells, and hematopoietic cells (25-30). A number of immunotherapy clinical trials targeting WT1 have been conducted to date. No clinically evident treatment-related toxicities have been observed in these trials (31-34). Multiple clinical trials in which patients with acute myelogenous leukemia are infused with autologous or allogeneic T cells redirected by HLA class I-restricted WT1-specific TCRs are currently ongoing.

The HLA-A*24:02/WT1235-243 (A24/WT1235)-specific TCR, clone TAK1, was previously isolated from an HLA-A24+ B*57:01 (B57)− donor. Further characterization revealed that TAK1 TCR indeed possesses cross-reactivity for unrelated B57 molecules (35). Using the TAK1 TCR as a model, we comprehensively studied the effects of TCR chain centricity on reactivity to target self-MHC/peptide complexes and alloreactivity.

Materials and Methods

Cells

Blood samples were obtained from healthy donors following institutional review board approval. HLA-A*24:02 positivity was determined using a PCR-based genotyping method (36). All donors were determined negative for B*57:01 by high resolution HLA DNA typing (American Red Cross). Mononuclear cells were obtained by density gradient centrifugation (Lymphoprep; Nycomed Pharma AS, Oslo, Norway). CD34+ hematopoietic cells were purified from cord blood mononuclear
cells using FITC-conjugated anti-CD34 monoclonal antibody (mAb) (clone AC136; Miltenyi Biotec) in combination with anti-FITC microbeads according to the manufacturer’s instruction (Miltenyi Biotec). We directly obtained the following cell lines from American Type Culture Collection (Manassas, VA): K562, which is an erythroleukemic cell line defective for HLA expression, and T2, which is a T-cell leukemia/B-cell large cell leukemia (B-LCL) hybrid cell line. Jurkat 76, devoid of endogenous TCR and CD8 expression, was kindly provided by Dr. Heemskerk, Leiden University Medical Centre (37). All cell lines were cultured as reported previously (38, 39) and passaged for less than 6 months.

cDNAs

Codon-optimized TAK1 TCR genes were produced by Thermo Fisher Scientific (Burlingame, CA) (35). For transduction into peripheral T cells, the TAK1 TCR hemi-chain was fused with a truncated form of nerve growth factor receptor (ΔNGFR) as described previously (20, 40). ΔNGFR gene alone was employed as a control. To clone TCRα genes, 5’-rapid amplification of cDNA ends (RACE) PCR was performed using SMARTer RACE cDNA amplification kit (Takara Bio). For the first-round of PCR, cDNA was amplified using a supplied 5’-RACE primer and a 3’-TCRα untranslated region primer (5’-GGAGAGTTCCCTCTGTTTGGAGAG-3’). The second-round PCR was performed using a modified 5’-RACE primer (5’-GTGTGGTGGTACGGGAATTCAAGCAGTGGTATCAACGCAGAGT-3’) and a 3’-TCRα primer (5’-ACCACTGTGCTGGCGGCCGCTCAGCTGGACCACAGCCGCACAGCG-3’). TCR genes fused with or without ΔNGFR were cloned into a pMX retrovirus vector and utilized to transduce cell lines and primary human T cells. Nucleotide sequencing was performed at the Centre for Applied Genomics, The Hospital for Sick Children (Toronto, Canada). TCR gene allele names are in accordance with IMGT unique gene nomenclature (http://www.imgt.org/).
Transfectants

Jurkat 76/CD8 cells stably express CD8α and β molecules (39). To generate TCR transfectants, Jurkat 76/CD8 cells were initially transduced with the TAK1β gene and then superinfected with individual TCRα genes. These TCR genes were not tagged with ΔNGFR gene. All TCR transfectants were purified using FITC-conjugated anti-TRBV5.1 mAb (clone IMMU 157; Beckman Coulter) in combination with anti-FITC microbeads according to the manufacturer’s instruction (Miltenyi Biotec). TRBV5.1 is encoded by the TAK1β gene. T2 cells were transduced with HLA-A24 to generate T2-A24. K562-based A24-artificial antigen-presenting cells (aAPC) and B57-aAPCs expressing HLA-A24 and B57 as a single HLA allele, respectively, in association with CD80 and CD83, were established as reported elsewhere (38, 39). PG13-derived retrovirus supernatants were utilized to transduce genes into T cells.

Expansion of TCR hemi-chain-transduced T cells

Peripheral T cells were stimulated with 50 ng/mL anti-CD3 mAb (clone OKT3) and 100 IU/mL human IL2 (Novartis) for 3 days and retrovirally transduced with TAK1α or β gene fused with ΔNGFR or with ΔNGFR alone (control). CD8+ T cells were purified using the CD8+ T Cell Isolation Kit (Miltenyi Biotec) and subsequently expanded using aAPCs as described previously (38, 39, 41). Briefly, CD8+ T cells were plated at 2 x 10^6 cells/well in RPMI 1640 supplemented with 10% human AB serum. Where indicated, A24-aAPCs was pulsed with 1 μg/ml A24-restricted WT1_{235-243} peptide (GenWay Biotech) for 6 hrs at room temperature and irradiated at 200 Gy before use. B57-aAPCs were always used without any peptide pulse. Starting the next day, 10 IU/ml IL2 (Novartis) and 10 ng/ml IL15 (Peprotech) were added to the cultures every three days. T cells were harvested, counted,
and restimulated every week. T-cell analysis was performed one day prior to or on the day of
restimulation.

**Flow cytometry analysis**

Cell surface molecules were stained with PC5-conjugated anti-CD8 mAb (clone B9.11,
Beckman Coulter), FITC-conjugated anti-NGFR mAb (clone ME20.4; Biolegend), APC-conjugated
anti-CD107a mAb (clone H4A3; Biolegend), PE-conjugated anti-CD3 mAb (clone UCHT1; Becton
Dickinson), and FITC-conjugated anti-TRBV5.1 mAb (Beckman Coulter). Stained cells were analyzed
with flow cytometry (BD Biosciences), and data analysis were performed using FlowJo Version 7.6.4
software (TreeStar) as described previously (39).

**Tetramer staining**

Biotinylated HLA-A24/WT1235-243 monomers and A24/Survivin80-88 monomers were kindly
provided by the NIH Tetramer Core Facility. The monomers were multimerized using PE-conjugated
streptavidin (Thermo Fisher Scientific) and utilized as described previously (38, 39, 41).
A24/Survivin80 tetramer was always used as a control. Where indicated, data shown are gated on
ΔNGFR+ cells. Structural avidity was determined by staining with graded concentrations of
A24/WT1235 tetramer (20).

**ELISPOT analysis**

Cytokine ELISPOT assays were conducted as described previously (38, 39, 41). Briefly, T
cells were incubated with 2 x 10^4 per well of indicated aAPCs, T2-A24 cells, or 3 x 10^4 per well of
CD34+ cells in the presence or absence of peptide for 20-24 hrs at 37°C. HLA-A24-restricted HIV
env584-592 peptide was used as a control. Functional avidity was studied using T2-A24 cells pulsed with
graded concentrations of A24/WT1235 peptide as stimulators as reported previously (20, 39).
Cytotoxicity assay

Standard cytotoxicity assay was conducted as described previously (38, 39). Briefly, $^{51}$Cr-labeled $5.0 \times 10^3$ indicated aAPCs or T2-A24 cells loaded with peptides were mixed with gene-modified CD$^8^+$ T cells for 6 hrs at 37°C in a 96-well round-bottom plate. A24/HIV env$_{584}$ peptide was used as a control. Percent specific lysis was calculated using the following formula: $([\text{experimental result} - \text{spontaneous release}] / [\text{maximum release} - \text{spontaneous release}]) \times 100\%$.

In silico sequence analysis

The ScanProsite tool (http://prosite.expasy.org/scanprosite/) was used to search for human-derived peptide sequences containing critical amino acid residues recognized by A24/WT1$_{235}$ TCRs within the entire UniProtKB/Swiss-Prot database (release 2015_02 of 04-Feb-15 with 547,599 entries).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0b. To determine whether two groups were significantly different for a given variable, analysis was performed using the Welch’s $t$ test (two-sided). Comparative analyses between three or more different groups were achieved using repeated-measures ANOVA with the Greenhouse-Geisser correction, followed by Tukey’s multiple comparison test. $P$ values $< 0.05$ were considered statistically significant. Pearson’s correlation coefficients were utilized to assess the correlation between two independent variables. Values of $r \geq 0.7$ were considered correlated.

Results

TAK1β hemi-chain has a dominant role in A24/WT1$_{235}$ reactivity.
To investigate whether the TAK1α (TRAV20*02/TRAJ33*01) or β (TRBV5-1*01/TRBJ2-1*01) chain has a dominant role in A24/WT1\textsubscript{235} reactivity, peripheral T cells from four A24\textsuperscript{+} and two A24\textsuperscript{-} donors were retrovirally transduced with TAK1α or β hemi-chain or a control gene (ΔNGFR alone). To mark hemi-chain-transduced T cells, each hemi-chain gene was fused to the ΔNGFR gene as stated in the Materials and Methods. Following transduction and prior to stimulation, A24/WT1\textsubscript{235} tetramer-positive cells were detectable in TAK1β, but not TAK1α, hemi-chain-transduced CD8\textsuperscript{+} T cells in two of the four A24\textsuperscript{+} donors and one of the two A24\textsuperscript{-} donors (Supplementary Fig. S1). We previously reported on the A24-aAPCs, which can expand HLA-A24-restricted antigen-specific T cells (42). To further confirm that the observed A24/WT1\textsubscript{235} tetramer-positive cells were specific to A24/WT1\textsubscript{235} peptide and not cross-reactive to the self-HLA complex, CD8\textsuperscript{+} T cells were isolated and stimulated twice with A24-aAPCs loaded with A24/WT1\textsubscript{235} peptide. In all 6 donors tested, A24/WT1\textsubscript{235}-specific TAK1β-transduced CD8\textsuperscript{+} T cells demonstrated significantly increased A24/WT1\textsubscript{235} tetramer positivity compared with TAK1α or control transfectants (Fig. 1A, left and right).

TAK1β-transduced but not TAK1α-transduced T cells recognized exogenously pulsed A24/WT1\textsubscript{235} peptide in both IFNγ ELISPOT (Fig. 1B, top) and standard killing assays (Fig. 1B, bottom), further confirming the A24/WT1\textsubscript{235} specificity of TAK1β-transduced T cells. The parental cell line of the aAPCs, K562, endogenously expresses WT1 protein. It has been demonstrated that K562 expresses normal proteasome machinery and can naturally process and present HLA class I-restricted peptides derived from endogenous antigens such as WT1 (35, 41, 43). TAK1β-transduced but not TAK1α-transduced T cells were able to recognize naturally processed and presented A24/WT1\textsubscript{235} peptide in both IFNγ ELISPOT analysis (Fig. 1C, top) and a standard cytotoxicity assay (Fig. 1C, bottom). However, the recognition of endogenously processed and presented A24/WT1\textsubscript{235} peptide was not as robust as exogenously pulsed A24/WT1\textsubscript{235} peptide. These results demonstrate that TAK1β but not TAK1α hemi-chain has a dominant role in dictating A24/WT1\textsubscript{235} specificity and that a
fraction of TAK1β-transduced T cells likely possess functional avidity sufficient to recognize endogenously processed and presented A24/WT1235 peptide.

**TCRα chain repertoires reactive for A24/WT1235 and alloreactive for B57 in conjunction with TAK1β chain partially but incompletely overlap.**

As we published previously, the TAK1 TCR cross-reacts with unrelated B57 molecules expressed in blood cells (35). We next investigated whether TAK1α or β hemi-chain plays a dominant role in B57 allo-recognition. Similar to the description above in Fig. 1, peripheral T cells from four A24⁺ B57⁻ and two A24⁻ B57⁻ donors were transduced with TAK1α or β chain fused with ΔNGFR or a control (ΔNGFR alone) gene. CD8⁺ T cells were purified and subsequently stimulated with A24-aAPCs pulsed with A24/WT1235 peptide. After two stimulations, TAK1β-transduced but not TAK1α-transduced T cells from all 6 donors demonstrated A24/WT1235 specificity on IFNγ ELISPOT analysis (Fig. 2A, top). Furthermore, TAK1β transfectants consistently possessed enhanced B57 alloreactivity compared with endogenous B57 alloreactivity presented by control transfectants (Fig. 2A, bottom). Note that all the donors were B57-negative and that various levels of endogenous alloreactivity to B57 molecules were observed in the 6 donors. Because the identity of the B57-bound peptide that is recognized by TAK1 TCR is unknown, we were not able to stain the expanded T cells with B57 tetramer. Importantly, there was no correlation between the observed A24/WT1235-specific and B57-specific IFNγ production capabilities of the TAK1β-transduced T cells derived from the 6 donors (Fig. 2B).

Next, peripheral T cells from two A24⁺ B57⁻ donors and one A24⁻ B57⁻ donor were transduced with a control (ΔNGFR alone) gene or with TAK1α or β chain fused with ΔNGFR. CD8⁺ T cells were purified and stimulated with unpulsed B57-aAPCs. No A24-restricted stimulation was performed. Following two stimulations, A24/WT1235 tetramer-positive TAK1β-transduced but not TAK1α-
Transduced CD8⁺ T cells expanded in all 3 donors tested (Fig. 2C, left and right). Collectively, these data suggest that TAK1β but not TAK1α hemi-chain plays a dominant role in B57 allo-recognition as well as in A24/WT1235 recognition as shown in Fig. 1. Moreover, TCRα chain repertoires reactive for A24/WT1235 and alloreactive for B57 in conjunction with TAK1β chain appear to overlap partially but not completely.

**The TCRα chain repertoire that is reactive with A24/WT1235 and/or B57 is diverse.**

The results above suggested that a TCRα chain(s) that possesses A24/WT1235 reactivity but lacks B57 alloreactivity (and vice versa) likely exists. We isolated and characterized a panel of TCRα chain genes that harbored various degrees of A24/WT1235 specificity and/or B57 alloreactivity when paired with the TAK1β chain. Following retroviral transduction of the TAK1β gene fused with the ΔNGFR⁺ gene, CD8⁺ T cells were purified as described in Figs. 1-2 and stimulated with one of three aAPCs, A24-aAPCs loaded with or without A24/WT1235 peptide or unpulsed B57-aAPCs. T cells that were A24/WT1235 tetramer positive or CD107a positive upon stimulation with A24-aAPCs loaded with A24/WT1235 peptide or B57-aAPCs were collected (44). Of the collected T cells, we molecularly cloned 45 unique TCRα genes and determined their sequences (Fig. 3). The isolated TCRα genes utilized TRAV12-2, 20, 36, or 38-2 and encoded variable complementarity-determining region (CDR)3α amino acid sequences and lengths (Fig. 3, top). Jα usage was also diverse without any notable bias (Fig. 3, bottom). CDR3α sequences of the 45 cloned TCRα genes are shown in Table 1.

**A24/WT1235 reactivity and B57 alloreactivity are separable.**

Next, we individually reconstituted all 45 unique TCRα genes along with the TAK1β chain in Jurkat 76/CD8 cells. Parental Jurkat 76 cells are devoid of surface CD3 expression because of the lack of endogenous TCRαβ gene expression (37). As shown in Supplementary Fig. S2, all 45 Jurkat
76/CD8 TCR transfectants demonstrated similarly upregulated surface CD3 expression (>95%), confirming comparable expression levels of the transduced TCR genes.

We then functionally determined the A24/WT1\textsubscript{1235} reactivity and B57 alloreactivity of the 45 distinct TCRs reconstituted in Jurkat 76/CD8 cells (Table 1). All reconstituted TCRs successfully recognized A24/WT1\textsubscript{1235} and/or B57 molecules. We classified the 45 TCR\textalpha chains into three groups based on their reactivity to A24/WT1\textsubscript{1235} and/or B57 molecules: reactive to both A24/WT1\textsubscript{1235} and B57, reactive to A24/WT1\textsubscript{1235} alone, or reactive to B57 alone. Representative data for three distinct TCR transfectants are depicted in Fig. 4A. When paired with the TAK1\textbeta chain, TCR\textalpha chain Cl. A262 reacted with both A24/WT1\textsubscript{1235} and B57. In contrast, Cl. T4 and Cl. T262 recognized only B57 and A24/WT1\textsubscript{1235}, respectively, but not the other ligand. Interestingly, only two clones, Cl. T4 and Cl. T3, possessed B57 alloreactivity without A24/WT1\textsubscript{1235} reactivity, suggesting that the majority of B57 alloreactive TCRs composed with TAK1\textbeta inherently harbor A24/WT1\textsubscript{1235} reactivity (Table 1).

Twenty-one of the 45 cloned TCR\textalpha genes were able to recognize naturally processed and presented A24/WT1\textsubscript{1235} and/or B57 molecules (Fig. 4B, left). Intriguingly, among the 21 TCR\textalpha genes, TRAV36 TCR\textalpha chains (n=8) demonstrated very well correlated A24/WT1\textsubscript{1235} reactivity and B57 alloreactivity (Fig. 4B, center). In contrast, non-TRAV36 TCR\textalpha chains (n=13) harbored either A24/WT1\textsubscript{1235} reactivity or B57 alloreactivity but not both (Fig. 4B, right). These results suggest that A24/WT1\textsubscript{1235}-specific TRAV36 TCR\textalpha chains may possess essential B57 alloreactivity. Interestingly, five of eight TRAV36 TCR\textalpha chains contained TRAJ52 (Table 1). In contrast, non-TRAV36 TCR\textalpha chains demonstrated various magnitudes of B57 alloreactivity that did not correlate with their A24/WT1\textsubscript{1235} reactivity. These results indicate that \alpha\beta TCR reactivity to target self-MHC/peptide complexes and cross-reactivity to unrelated MHC molecules are not inextricably linked and are separable at the TCR sequence level.
TCRα chain broadly regulates T-cell avidity for A24/WT1235 when paired with chain-centric TAK1β.

As shown above, the TCRα counter-chain regulates both A24/WT1235 reactivity and B57 alloreactivity when paired with the chain-centric TAK1β chain. To compare structural avidity of Jurkat 76/CD8 TCR transfectants, we stained them with a suboptimal amount (5 μg/mL) of A24/WT1235 and A24/Survivin80 (control) tetramers (Fig. 5A and Supplementary Fig. S3). As shown in Supplementary Fig. S2, the CD3 positivity of all transfectants was uniformly greater than 95%, confirming that all individually transduced TCR genes were similarly reconstituted on the cell surface. Note that TCRα Cl. T4 paired with TAK1β was not stained with A24/WT1235 tetramer, since this transfectant was cross-reactive for B57 but not A24/WT1235 (Figs. 4A, 5A, and Table 1). Some clones, such as Cl. A54, Cl. T125, and Cl. T364, that were functionally reactive for A24/WT1235 similarly, did not stain well with the amount of tetramer used because of their low structural avidities (Supplementary Fig. S3 and Table 1).

To compare the structural avidities of the Jurkat 76/CD8 TCR transfectants shown in Fig. 5A in more detail, they were stained with graded concentrations of A24/WT1235 tetramer (Fig. 5B, left). To determine functional avidity, IL2 ELISPOT analysis was conducted using T2-A24 cells loaded with graded concentrations of A24/WT1235 peptide as stimulators (Fig. 5B, right). As shown in Fig. 5C, there was a positive correlation between the structural and functional avidities (EC50 in μg/mL).

Moreover, Jurkat 76/CD8 transfectants individually expressing various A24/WT1235 TCRα chains in association with TAK1β possessed a broad range (>3 log orders) of structural and functional avidities. Taken together, our results demonstrate that the chain centricity of the TAK1β chain can be exploited to isolate A24/WT1235 TCRs with structural and functional avidities over a broad range.
Newly isolated A24/WT1235 TCRs possess potential cross-reactivity to homologous peptides but lack reactivity to CD34+ hematopoietic cells.

Recent clinical trials have demonstrated that cross-reactivity of antitumor TCRs to other peptides presented by self-HLA molecules can cause unwanted serious adverse events in patients treated with TCR gene therapy (15, 16). Alanine substitution scanning identified that the first, second, eighth, and ninth amino acid residues of the A24/WT1235 peptide are critical for recognition by three different TCRs, Cl. T262, Cl. A262, and Cl. T53, which have sufficient affinity to recognize naturally processed and presented A24/WT1235 peptide (Fig. 6A). Based on this, an in silico search was conducted to identify protein sequences that contain the “CMXXXXXNL” sequence using the ScanProsite tool (45, 46). We synthesized eleven human-derived peptides homologous to A24/WT1235 as shown in Supplementary table S1. Jurkat 76/CD8 TCR transfectants, Cl. T262, Cl. A262, and Cl. T53, reacted to some of these peptides with different specificity and potency (Fig. 6B). It should be noted, however, that it is currently unknown whether these peptides are naturally processed and presented by A24 in normal tissues.

It is known that WT1 is expressed in human primary CD34+ hematopoietic cells (27, 30). We studied the reactivity of two Jurkat 76/CD8 TCR transfectants, Cl. T262 and Cl. A262 to purified CD34+ cells. Neither TCR transfectant showed any reactivity to unpulsed A24+ CD34+ cells. In contrast, both transfectants recognized A24/WT1235 peptide exogenously pulsed into A24+ CD34+ cells but not into A24- CD34+ cells (Fig. 6C). Collectively, our results suggest that, although newly cloned A24/WT1235 TCRs, Cl. T262 and Cl. A262, possess potential cross-reactivity to A24/WT1235-related peptides, they did not recognize human primary HLA-A24+ CD34+ hematopoietic cells.

Discussion
The TAK1α chain encodes TRAV20*02 and recognizes both the A24/WT1235 and B57 molecules when paired with TAK1β chain. Although TCRα chain Cl. T262, which has been newly cloned in this study, utilizes the same TRAV20*02, it reacts with A24/WT1235 but not with B57 molecules. This separation obviously suggests that the hypervariable CDR3α sequences affect the structure of the TCR, thereby regulating antigen specificity as well as alloreactivity in a separable manner and that self A24-restricted WT1235 specificity and B57 cross-reactivity are not inextricable.

Previous crystallographic studies have demonstrated that, in addition to the hypervariable CDR3 loop, germline-encoded variable CDR1/2 regions can play important roles in the recognition of MHC/peptide complexes (47, 48). Dai and colleagues demonstrated that amino acid substitutions in the variable CDR1/2 regions of murine YAe62 TCRs resulted in an alteration of alloreactivity for unrelated MHC/peptide complexes (49). In line with these studies, we showed that, among the 21 TAK1α chains that recognized naturally processed and presented A24/WT1235 and/or B*57:01 peptides, 8 TRAV36 TCRα counter-chains harbored well-correlated A24/WT1235 reactivity and B57 alloreactivity. TRAJ52 may be involved in the correlated A24/WT1235 and B57 reactivity in association with the TRAV36 when paired with TAK1β. In contrast, the remaining 13 non-TRAV36 TCRα chains demonstrated either exclusive A24/WT1235 reactivity or B57 alloreactivity depending upon their CDR3α sequences (Fig. 4). These findings indicate that, in concert with CDR3α loops, TCR CDR1/2α regions can affect both A24/WT1235 reactivity and B57 cross-reactivity when paired with the chain-centric TAK1β chain.

Although further research is required, the results obtained using functional assays imply that the TAK1β hemi-chain has a dominant role in dictating B57 cross-reactivity (Fig. 2). It is thus highly likely that TAK1β has a dominant role in dictating both A24/WT1235 reactivity and B57 cross-reactivity. A TCR with similar attributes has been reported previously. Crystallographic analysis of another chain-centric TCR, clone LC13 that is HLA-B8/EBV EBNA3A339-specific and B44
alloreactive, has demonstrated that it recognizes both B8/EBV EBNA3A339 and B44 using similar conformation (10). Because isolated TRAV36 TCR\(\alpha\) chains possessed well-correlated A24/WT1\textsubscript{235} reactivity and B57 alloreactivity when paired with TAK1\(\beta\), they may recognize A24/WT1\textsubscript{235} and B57 with a similar footprint (Fig. 4B). In contrast, non-TRAV36 TCR\(\alpha\) clonotypes, which possess either A24/WT1\textsubscript{235} reactivity or B57 alloreactivity exclusively, may bind A24/WT1\textsubscript{235} or B57 using different conformations. Crystallographic analysis of these distinct TCR\(\alpha\) chains paired with the TAK1\(\beta\) chain is warranted.

Multiple clonotypic TCR\(\alpha\) counter-chains were able to recognize A24/WT1\textsubscript{235} and/or B57 when paired with TAK1\(\beta\), and these TCRs possessed a very wide range (>3 log orders) of structural and functional avidities for A24/WT1\textsubscript{235}. Of note, TCR\(\alpha\) hemi-chains such as Cl. A262 and Cl. T262 were able to recognize naturally processed and presented A24/WT1\textsubscript{235} peptide when reconstituted with TAK1\(\beta\) on Jurkat 76/CD8 cells, suggesting that they possess sufficient affinity to recognize tumor cells in the presence of CD8 co-receptors (Table 1). When reconstituted along with TAK1\(\beta\) in the absence of CD8 molecules, however, none of the isolated TCR\(\alpha\) chains were stained with A24/WT1\textsubscript{235} tetramer or recognized naturally processed and presented A24/WT1\textsubscript{235} peptide (data not shown). High-affinity HLA class I-restricted TCRs that can recognize endogenously processed and presented peptides in the absence of CD8 co-engagement do exist and have been isolated for various antigens (50). We previously reported that T cells with higher avidities preferentially grew from A2-restricted antigen-specific CD8\(^+\) T cells that had been stimulated with aAPCs expressing mutated HLA-A2 molecules that cannot interact with CD8 molecules (39). Stimulation of TAK1\(\beta\)-transduced CD8\(^+\) T cells with aAPCs expressing mutated A24 that cannot associate with CD8 may enrich TAK1\(\beta^+\) A24/WT1\textsubscript{235} CD8\(^+\) T cells with higher avidity and facilitate the cloning of high-affinity TCR\(\alpha\) chains that do not require CD8 co-engagement to recognize target cells (39).
The selection of TCRs with minimal on-target/off-tumor and off-target toxicities is critical to conduct safe TCR gene therapy (5, 7). We have demonstrated that newly isolated A24/WT1\textsubscript{235} TCRs do not recognize A24\textsuperscript{−}CD34\textsuperscript{+} hematopoietic cells (Fig. 6C). And yet, since WT1 is expressed in other normal cells such as podocytes in the kidney and mesothelial cells (26, 28), potential toxicity to these tissues needs to be carefully monitored in any immunotherapy, especially adoptive T-cell therapy targeting WT1. Off-target adverse events associated with the infusion of redirected T cells are thought to be due, at least partly, to the cross-reactivity of the transduced TCR itself or to mispaired TCRs. In fact, severe adverse events associated with the infusion of redirected T cells that are cross-reactive for other antigens have been reported (15, 16). We have shown that it is possible to eliminate inherent alloreactivity from antigen-specific TCRs at the molecular level by exploiting TCR chain centricity. However, newly cloned A24/WT1\textsubscript{235} TCRs containing TAK1\beta chain possessed potential cross-reactivity to peptides homologous to A24/WT1\textsubscript{235}, although it is unknown whether these peptides are endogenously processed and presented by A24 in normal tissues (Fig. 6B). These findings suggest that we cannot eliminate the possibility that TCRs lacking apparent cross-reactivity may still acquire \textit{de novo} cross-reactivity for self/non-self HLA complexes. However, preparing a set of TCRs with the same antigen specificity and different cross-reactivity by exploiting TCR chain centricity would benefit cancer patients by enlarging the patient population that can be treated using TCR gene therapy.

Acknowledgements

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References


47. Garcia KC. Reconciling views on T cell receptor germline bias for MHC. Trends Immunol 2012;33:429-36.


Table 1. Variable, joining region, and CDR3\(\alpha\) sequences of TCR\(\alpha\) clonotypes and their reactivities to unloaded A24-aAPC, B57-aAPC, and A24-aAPC loaded with A24/WT1235 peptide

<table>
<thead>
<tr>
<th>Clone</th>
<th>TRAV</th>
<th>CDR3(\alpha)</th>
<th>TRAJ</th>
<th>Targets</th>
<th>Unloaded A24-aAPC</th>
<th>Unloaded B57-aAPC</th>
<th>A24-aAPC loaded with A24/WT1235 peptide</th>
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</thead>
<tbody>
<tr>
<td>TAK1(\alpha)</td>
<td>20*02</td>
<td>CAVQAVDSNYQILW</td>
<td>33*01</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td></td>
</tr>
</tbody>
</table>

**TCR\(\alpha\) chains isolated following stimulation with A24-aAPC loaded with A24/WT1235 peptide**

| Cl | 20*02 | CAVQAVDSNYQILW | 33*01 | - | ++ | +++ |

**TCR\(\alpha\) chains isolated following stimulation with unloaded A24-aAPC**

| Cl | 20*02 | CAVQAVDSNYQILW | 33*01 | - | ++ | +++ |

**TCR\(\alpha\) chains isolated following stimulation with unloaded B57-aAPC**

A24/WT1235 reactivity and B57 alloreactivity of 45 Jurkat 76/CD8 TCR transfectants were measured using A24-aAPCs loaded with or without 1 \(\mu\)g/mL A24/WT1235 peptide and B57-aAPCs as stimulators in IL-2 ELISPOT assays. All the experiments were performed in triplicate, and the mean number of IL-2 SFUs per \(4.0 \times 10^4\) cells was calculated. +++: >400 SFU/\(4.0 \times 10^4\) cells; ++: 101-400 SFU/\(4.0 \times 10^4\) cells; +: 10-100 SFU/\(4.0 \times 10^4\) cells; -: <10 SFU/\(4.0 \times 10^4\) cells. All the transfectants produced <5 SFU per \(4.0 \times 10^4\) cells when stimulated with HLA-null aAPCs (data not shown).
Figure legends

Figure 1. The TAK1β hemi-chain has a dominant role in dictating A24/WT1235 reactivity. A, TAK1β but not TAK1α hemi-chain-transduced CD8+ T cells are positively stained with A24/WT1235 tetramer following antigen-specific stimulation. Peripheral T cells from four A24+ donors and two A24- donors were retrovirally transduced with a control gene (ΔNGFR alone) or TAK1 hemi-chain fused with ΔNGFR. CD8+ T cells were isolated and stimulated weekly with A24-aAPCs loaded with 1 μg/mL A24/WT1235 peptide. After two stimulations, the expanded T cells were stained with A24/WT1235 or A24/Survivin80 (control) tetramer in conjunction with anti-CD8 mAb and anti-NGFR mAb. Data shown are gated on ΔNGFR+ cells. Representative data for one A24+ donor (Donor 1) and one A24- donor (Donor 2) are shown (left). The A24/WT1235 tetramer positivities of the hemi-chain-transduced T cells from six different donors were compared (right). B, TAK1β- but not TAK1α-transduced CD8+ T cells recognize exogenously pulsed A24/WT1235 peptide. TAK1 hemi-chain+ CD8+ T cells were expanded as described in (A) and subjected to IFN-γ ELISPOT analysis. T2-A24 cells pulsed with 10 μg/mL A24/HIV env584 (control) or A24/WT1235 peptide were used as stimulator cells (top). Data shown were obtained from A24+ Donor 1 and A24- Donor 2. Standard cytotoxicity assays were also conducted utilizing TAK1 hemi-chain+ CD8+ T cells derived from Donor 1 and T2-A24 cells pulsed with the indicated peptides (bottom). All the experiments were conducted in triplicate, and error bars depict SD. C, TAK1β but not TAK1α-transduced CD8+ T cells recognize naturally processed and presented A24/WT1235 peptide. TAK1 hemi-chain+ CD8+ T cells expanded as described in (A) were subjected to IFN-γ ELISPOT and killing assays as shown in (B). Unpulsed A24-aAPCs were used as target cells. HLA-null aAPCs, which lack the expression of HLA molecules, were employed as a control. All the experiments were performed in triplicate, and error bars demonstrate the SD. *p < 0.05, **p < 0.01. n.s., not significant.
**Figure 2. TAK1α repertoires specific for A24/WT1235 and alloreactive for B57 partially overlap.**

A, TAK1β but not α-transduced CD8+ T cells have enhanced alloreactivity for HLA-B57. Peripheral T cells from four A24+ donors and two A24- donors were retrovirally transduced with TAK1α or β fused with ΔNGFR or a control gene (ΔNGFR alone). All the donors were B57 negative. CD8+ T cells were isolated and stimulated twice with A24-aAPCs loaded with 1 μg/mL A24/WT1235 peptide. IFN-γ ELISPOT analysis was conducted using A24-aAPCs loaded with 1 μg/mL A24/HIV env584 peptide (control) or A24/WT1235 peptide (top) and HLA-null (control) or B57-aAPCs (bottom). Representative data for A24+ Donor 1 and A24- Donor 2 are shown. All the experiments were conducted in triplicate, and error bars show the SD. B, A24/WT1235 reactivity and B57 alloreactivity of TAK1β-transduced CD8+ T cells are not correlated. TAK1β-transduced CD8+ T cells were generated as described in (A). IFN-γ secretion capabilities of TAK1β-transduced CD8+ T cells against A24-aAPCs loaded with 1 μg/mL A24/WT1235 peptide and B57-aAPCs were compared in 6 different donors using ELISPOT assays. Each dot represents one donor. C, TAK1β but not α-transduced CD8+ T cells became specific for A24/WT1235 following stimulation with B57-aAPCs. Peripheral T cells from two A24+ donors and one A24- donor were retrovirally transduced with TAK1α or β fused with ΔNGFR or a control gene (ΔNGFR alone). CD8+ T cells were isolated and stimulated weekly with B57-aAPCs. After two stimulations, the gene-modified CD8+ T cells were stained with A24/WT1235 or A24/Survivin80 (control) tetramer in conjunction with anti-CD8 mAb and anti-NGFR mAb. Data shown are gated on ΔNGFR+ cells. Representative data for A24+ Donor 1 and A24- Donor 2 are shown (left). The A24/WT1235 tetramer positivity of the T cells from three different donors was compared (right). Note that the data points shown for all three donors partially overlap. *p < 0.05, **p < 0.01, ***p < 0.001.

n.s., not significant.
Figure 3. TCRα clonotypes specific for A24/WT1_{235} and/or alloreactive for B57 when paired with the TAK1β chain are highly heterogeneous. CD8+ T cells were purified from three A24+ donors and one A24- donor and transduced with the TAK1β gene. The hemi-chain+ T cells were stimulated with A24-aAPCs loaded with or without A24/WT1_{235} peptide or unpulsed B57-aAPCs. A24/WT1_{235} tetramer-positive cells or CD107a-positive T cells stimulated with A24-aAPCs loaded with A24/WT1_{235} peptide or B57-aAPCs were collected by fluorescence-activated cell sorting. TCRα genes were cloned from the collected T cells, and their sequences were determined. Forty-five different TCRα clonotypes specific for A24/WT1_{235} and/or alloreactive for B57 were isolated (see also Table 1). Cloned TCRα genes encoded TRAV12-2, TRAV20, TRAV36, or TRAV38-2. CDR3α lengths (top) and Jα region usage (bottom) of the cloned TCRα genes are depicted based on TRAV usage.

Figure 4. B57 alloreactivity is separable from A24/WT1_{235} reactivity by changing the TCRα chains paired with the TAK1β chain. A, Cloned TCRα chains demonstrate A24/WT1_{235} reactivity and/or B57 alloreactivity when reconstituted with the TAK1β chain on Jurkat 76/CD8 cells. The A24/WT1_{235} reactivity and B57 alloreactivity of all 45 Jurkat 76/CD8 TCR transfectants were measured using the indicated aAPCs as stimulators in IL-2 ELISPOT assays (Table 1). The A24/WT1_{235} reactivity and B57 alloreactivity of three representative TCRα clones, Cl. A262, Cl. T4, and Cl. T262, are shown. All the experiments were conducted in triplicate, and error bars depict the SD. Data shown are representative of two independent experiments. B, The A24/WT1_{235} reactivity and B57 alloreactivity of TRAV36 but not non-TRAV36 TCRα genes correlated. The A24/WT1_{235} reactivity and B57 alloreactivity of 21 TCRα chains (Table 1) that recognize naturally processed and presented A24/WT1_{235} peptide and/or B57 molecules when paired with TAK1β were compared (left). The 21 TCRα chains were divided into 2 groups, 8 TRAV36 TCRα (center) and 13 non-TRAV36
TCRα genes (right). Each dot represents one donor. Pearson’s correlation coefficients were calculated; r and p values are shown.

**Figure 5. Structural and functional avidity ranges of Jurkat 76/CD8 cells expressing A24/WT1\textsubscript{235} TCRs composed with the TAK1\textbeta hemi-chain are very broad.**

**A,** Clonotypic TCRα chains reconstituted along with the TAK1\textbeta gene on Jurkat 76/CD8 cells were differentially stained by A24/WT1\textsubscript{235} tetramer. All forty-five Jurkat 76/CD8 TCR transfectants were stained with a suboptimal concentration (5 μg/mL) of A24/WT1\textsubscript{235} or A24/Survivin\textsubscript{80} tetramer in conjunction with anti-CD8 mAb. Six representative clones that display different structural avidities are shown. Jurkat 76/CD8 cells expressing the TAK1\textbeta gene alone, TCRα (-), were used as a negative control. Data for the remaining thirty-nine transfectants are shown in Supplementary Fig. S3. All the transfectants showed >95% CD3 positivity, confirming similar expression levels for transduced TCRs (Supplementary Fig. S2). The TCRα gene Cl. T4 was reactive for only B57 but not A24/WT1\textsubscript{235} (Fig. 4A). Data shown are representative of two independent experiments. **B,** Jurkat 76/CD8 T cells reconstituted with cloned TCRα genes along with the TAK1\textbeta possess a broad range of structural and functional avidities. Structural avidity of Jurkat 76/CD8 transfectants individually expressing the five different TCRα chains including the original TAK1\textalpha chain in (A) in association with the TAK1\textbeta chain are depicted as percentages of maximal tetramer staining intensity determined by staining with graded concentrations of A24/WT1\textsubscript{235} tetramer (left). Functional avidity of the same transfectants are shown as % IL-2 secreting ability as determined by IL-2 ELISPOT assays using T2-A24 cells pulsed with graded concentrations of A24/WT1\textsubscript{235} peptide as a stimulator (right). The analysis was conducted in triplicate, and error bars show the SD. **C,** Structural and functional avidities for A24/WT1\textsubscript{235} determined in (B) strongly correlated in the 5 Jurkat 76/CD8 TCR transfectants tested. Structural avidity, expressed as the EC\textsubscript{50} in μg/mL, was defined as the concentration of A24/WT1\textsubscript{235} tetramer required to achieve 50%
of maximal staining intensity. Similarly, functional avidity, shown as the EC₅₀ in μg/mL, was determined by the concentration of A24/WT1₂₃₅ peptide required to achieve 50% of maximal response. Each dot represents one donor. Pearson’s correlation coefficients were calculated; r and p values are shown.

**Figure 6.** Newly cloned A24/WT1₂₃₅ TCRs possess potential cross-reactivity to homologous peptides but lack reactivity to CD34⁺ hematopoietic cells. A, Alanine substitution analysis identified A24/WT1₂₃₅ peptide residues important for recognition by A24/WT1₂₃₅ TCRs. Each residue in the A24/WT1₂₃₅ peptide sequence was sequentially replaced by an alanine residue. The reactivity of Jurkat 76/CD8 cells reconstituted with Cl. T262, Cl. A262, or Cl. T53 along with TAK1β is shown as % IL-2 maximum secretion capacity as determined by IL-2 ELISPOT assays using T2-A24 cells pulsed with 50 μg/mL parental wild-type A24/WT1₂₃₅ or each alanine substituted peptide. A24/HIV env₅₈₄ was used as a negative control. Note that these three Jurkat 76/CD8 transfectants possess functional avidity sufficient to recognize unpulsed A24-aAPCs (Table 1). The experiments were conducted in triplicate, and error bars depict the SD. *p < 0.05, **p < 0.01. B, A24/WT1₂₃₅ TCRs possess potential cross-reactivity to peptides homologous to wild-type A24/WT1₂₃₅. Jurkat 76/CD8 cells transfectants expressing Cl. T262, Cl. A262, or Cl. T53 were stimulated as in (A). The experiments were conducted in triplicate, and error bars show the SD. Data shown are representative of two independent experiments. Sequences of twelve A24/WT1₂₃₅-related peptides including the wild-type A24/WT1₂₃₅ peptide are shown in Supplementary table S1. C, A24/WT1₂₃₅-specific TCRs did not recognize human primary CD34⁺ hematopoietic cells. Two A24/WT1₂₃₅-specific Jurkat 76/CD8 transfectants, Cl. T262 and Cl. A262, were studied for their reactivity to human cord blood CD34⁺ cells purified from two A24⁺ and one A24⁻ donors. IL-2 ELISPOT assays were conducted using purified CD34⁺ cells as stimulator cells. CD34⁺ cells pulsed with 50 μg/mL A24/HIV env₅₈₄ or
A24/WT1235 peptide were used as a negative or positive control. The experiments were conducted in triplicate, and error bars show the SD.
Figure 1.

A

Transduced with

<table>
<thead>
<tr>
<th>Control</th>
<th>TAK1α</th>
<th>TAK1β</th>
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<tbody>
<tr>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>0.7%</td>
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<td></td>
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Survivin

WT129

CD8

% A24/WT129

CD8+ T cells

n=6

B

Number of IFN-γ SFU per 2x10^6 cells

Donor 1

Donor 2

* * *

Control TAK1α TAK1β

Specific cytotoxicity (%)

E:T

10:1 3:1 1:1

T2-A24 + A24/HIV em160 peptide

T2-A24 + A24/WT129 peptide

C

Number of IFN-γ SFU per 2x10^6 cells

Donor 1

Donor 2

n.s. **

Control TAK1α TAK1β

Specific cytotoxicity (%)

E:T

30:1 10:1 3:1

None

HLA-null-aAPC

A24-aAPC

HLA-null aAPC

A24-aAPC
Figure 2.

A

Donor 1

Donor 2

B

Number of loaded A24/WT1<sup>125C</sup>-specific IFN-γ SFU per 2x10<sup>4</sup> cells

Number of unloaded B57-specific IFN-γ SFU per 2x10<sup>4</sup> cells

C

Transduced with

Donor 1

Donor 2

Survivin<sub>10</sub>

WT1<sub>125C</sub>

CD8
Figure 3.
Figure 4.
Figure 5.

A

Transduced with TCRα

<table>
<thead>
<tr>
<th>Cl.T262</th>
<th>Cl.A262</th>
<th>TAK1α</th>
<th>Cl.A186</th>
<th>Cl.A133</th>
<th>CLT4</th>
<th>TCRα (-)</th>
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<td>85.5%</td>
<td>83.6%</td>
<td>54.2%</td>
<td>48.1%</td>
<td>7.4%</td>
<td>0.9%</td>
<td>0.7%</td>
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</table>

Jurkat 76/CD8/TAK1β

WT1235

Survivin

A24 tetramer

CD8

B

% maximal staining intensity

% IL-2 secreting ability

Concentrations of A24/WT1235 tetramer (µg/mL)

Concentrations of A24/WT1235 peptide (µg/mL)

C

r = 0.9999, p < 0.0001

Functional avidity (EC50 in µg/mL)

Structural avidity (EC50 in µg/mL)
Figure 6.

A

Transduced with TCRα

% IL-2 secreting ability

Cl.T262

Cl.A262

Cl.T53

Alanine-substituted positions

B

Transduced with TCRα

Number of IL-2 SFU per 1x10⁶ cells

Cl.T262

Cl.A262

Cl.T53

A24/WT1230-related peptides

C

Transduced with TCRα

Number of IL-2 SFU per 1x10⁶ cells

Cl.T262

Cl.A262

Donor 4 Donor 5 Donor 6

A24+ A24−

Donor 4 Donor 5 Donor 6

A24+ A24−

Donor 4 Donor 5 Donor 6

A24+ A24−

Donor 4 Donor 5 Donor 6

A24+ A24−

CD34+ cells

CD34+ cells

+A24/HIV env peptide

+A24/WT1230 peptide
Optimization of T-cell reactivity by exploiting TCR chain centricity for the purpose of safe and effective antitumor TCR gene therapy

Toshiki Ochi, Munehide Nakatsugawa, Kenji Chamoto, et al.

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