Optimization of T-cell Reactivity by Exploiting TCR Chain Centricity for the Purpose of Safe and Effective Antitumor TCR Gene Therapy

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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 affects reactivity to target self-MHC/peptide complexes and alloreactivity using the TCR, clone TAK1, which is specific for human leukocyte antigen-A24:24-26/HLA tumor 1235–243 (A24/WT1235) and cross-reactive with B57:01 (B57). The TAK1B, but not the TAK1C, hemi-chain possessed chain centricity. When paired with multiple clonotypic TCRtx 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 TCRx 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 impairs 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; refs. 3–7). These redirected T cells are endowed with specificity and strong reactivity for tumor cells by transducing high-affinity antitumor CAR or TCR genes (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 off-target adverse events, 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, because 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 hema-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, because 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-A24/WT1235-B57 (A24/WT1235) specific TCR, clone TAK1, was previously isolated from an HLA-A24+ 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-A24+ positivity was determined using a PCR-based genotyping method (36). All donors were determined negative for B57+ by high-resolution HLA DNA typing (American Red Cross). Mononuclear cells were obtained by density gradient centrifugation (Lymphoprep; Nycomed Pharma AS). CD34+ hematopoietic cells were purified from cord blood mononuclear cells using FITC-conjugated anti-CD34 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 the American Type Culture Collection: K562, which is an erythroleukemic cell line defective for HLA expression, and T2, which is a T-cell leukemia/B-cell large cell leukemia hybrid cell line. Jurkat 76, devoid of endogenous TCR and CD8 expression, was kindly provided by Dr. Heemskerk, Leiden University Medical Centre, Leiden, Netherlands (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 (35). For transduction into peripheral T cells, the TAK1 TCR hemi-chain was fused with a truncated form of nerve growth factor receptor (NGFR) as described previously (20, 40). ΔNGFR gene alone was employed as a control. To clone TCRγ genes, 5′-rapid amplification of cDNA ends (RACE) PCR was performed using a 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′-RACE primer untranslated region primer (5′-GGAGAATCGGCAGACCGGAG-3′). The second-round PCR was performed using a modified 5′-RACE primer (5′-GTTGCGCGGCCACCGTGTTGAGG-3′). For the NGFR gene, the following primers were used: 5′-ACACGCTTGGATCGGAGG-3′ and 3′-RACE primer (5′-ACACGCTTGGATCGGAGG-3′). TCR genes fused with or without NGFR 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 IMMUL157; 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 TAK1γ or β gene fusion 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 × 10^5 cells per well in RPMI 1640 supplemented with 10% human AB serum. Where indicated, A24-aAPCs were pulsed with 1 μg/mL A24-restricted WT1235-243 peptide (GenWay Biotech) for 6 hours 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 3 days. T cells were harvested, counted, and restimulated every week. T-cell analysis was performed 1 day before 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-NGFR mAb (clone ME20.4; Biolegend), APC-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 was performed using FlowJo Version 7.6.4 software (TreeStar) as described previously (39).

Tetramer staining

Biotinylated HLA-A24/WT1235-243 monomers and A24/Survivin0 α0 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/Survivin0 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 $2 \times 10^6$ per well of indicated aAPCs, T2-A24 cells, or $3 \times 10^7$ per well of CD34$^+$ cells in the presence or absence of peptide for 20 to 24 hours 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 CD8$^+$ T cells for 6 hours at 37°C in a 96-well round-bottom plate. A24/HIV enV584 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/WT1235 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 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 multiple comparison test. $P$ values $< 0.05$ were considered statistically significant. Pearson 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/WT1235 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/WT1235 reactivity, peripheral T cells from four A24$^+$ and two A24$^-$ 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 Materials and Methods. Following transduction and before stimulation, A24/WT1235 tetramer–positive cells were detectable in TAK1β, but not TAK1α, hemi-chain–transduced CD8$^+$ T cells in two of the four A24$^+$ donors and one of the two A24$^-$ 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/WT1235 tetramer–positive cells were specific to A24/WT1235 peptide and not cross-reactive to the self-HLA complex, CD8$^+$ T cells were isolated and stimulated twice with A24-aAPCs loaded with A24/WT1235 peptide. In all 6 donors tested, A24/WT1235–specific TAK1β–transduced CD8$^+$ T cells demonstrated significantly increased A24/WT1235 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/WT1235 peptide in both IFNγ ELISPOT (Fig. 1B, top) and standard killing assays (Fig. 1B, bottom), further confirming the A24/WT1235 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/WT1235 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/WT1235 peptide was not as robust as exogenously pulsed A24/WT1235 peptide. These results demonstrate that TAK1β, but not TAK1α, hemi-chain has a dominant role in dictating A24/WT1235 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$^+$ and two A24$^-$ 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 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.
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⁺/C0 donors were retrovirally transduced with a control gene (NGFR alone) or TAK1 hemi-chain fused with NGFR. 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 NGFR⁺ cells. Representative data for one A24⁺ donor (Donor 1) and one A24⁺/C0 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-transduced, 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 IFNγ 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⁺/C0 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 IFNγ 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 statistically significant.
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 TAKβ chain. Following retroviral transduction of the TAKβ gene fused with the NGFR gene, CD8+ T cells were purified as described in Figs. 1 and 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
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Figure 3.
TCRx clones specific for A24/WT1235 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/WT1235 peptide or unpulsed B57-aAPCs. A24/WT1235 tetramer-positive cells or CD107a-positive T cells stimulated with A24-aAPCs loaded with A24/WT1235 peptide or B57-aAPCs were collected by fluorescence-activated cell sorting. TRCX genes were cloned from the collected T cells, and their sequences were determined. Forty-five different TRCX clonotypes specific for A24/WT1235 and/or alloreactive for B57 were isolated (see also Table 1). Clone TCRx genes encoded TRAV12-2, TRAV20, TRAV36, or TRAV38-2. CDR3 lengths (top) and Jx region usage (bottom) of the cloned TCRx genes are depicted based on TRAV usage.

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 TCRx genes and determined their sequences (Fig. 3). The isolated TCRx genes utilized TRAV12-2, 20, 36, or 38-2 and encoded variable complementarity-determining region (CDR3) amino acid sequences and lengths (Fig. 3, top). Cx usage was also diverse without any notable bias (Fig. 3, bottom). CDR3x sequences of the 45 cloned TCRx genes are shown in Table 1.

A24/WT1235 reactivity and B57 alloreactivity are separable

Next, we individually reconstituted all 45 unique TCRx 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 TCRβ gene expression (37). As shown in Supplementary Fig. S2, all 45 Jurkat 76/CD8 TCRx transfectants demonstrated similarly upregulated surface CD3 expression (>95%), confirming comparable expression levels of the transduced TCRx genes.

We then functionally determined the A24/WT1235 reactivity and B57 alloreactivity of the 45 distinct TCRx reconstituted in Jurkat 76/CD8 cells (Table 1). All reconstituted TCRx successfully recognized A24/WT1235 and/or B57 molecules. We classified the 45 TCRx chains into three groups based on their reactivity to A24/WT1235 and/or B57 molecules: reactive to both A24/WT1235 and B57, reactive to A24/WT1235 alone, or reactive to B57 alone. Representative data for three distinct TCRx transfectants are depicted in Fig. 4A. When paired with the TAK1β chain, TCRx chain Cl. A262 reacted with both A24/WT1235 and B57. In contrast, Cl. T4 and Cl. T262 recognized only B57 and A24/WT1235, respectively, but not the other ligand. Interestingly, only two clones, Cl. T4 and Cl. T3, possessed B57 alloreactivity without A24/WT1235 reactivity, suggesting that the majority of B57 alloreactive TCRx chains were independent of A24/WT1235 reactivity (Table 1).

Twenty-one of the 45 cloned TCRx genes were able to recognize naturally processed and presented A24/WT1235 and/or B57 molecules (Fig. 4B, left). Intriguingly, among the 21 TCRx genes, TRAV36 TCRx chains (n = 8) demonstrated very well correlated A24/WT1235 reactivity and B57 alloreactivity (Fig. 4B, center). In contrast, non-TRAV36 TCRx chains (n = 13) harbored either A24/WT1235 reactivity or B57 alloreactivity but not both (Fig. 4B, right). These results suggest that A24/WT1235-specific TRAV36 TCRx chains may possess essential B57 alloreactivity. Interestingly, five of eight TRAV36 TCRx chains contained TRAJ52 (Table 1). In contrast, non-TRAV36 TCRx chains demonstrated various magnitudes of B57 alloreactivity that did not correlate with their A24/WT1235 reactivity. These results indicate that singleTCRx 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.

TCRx chain broadly regulates T-cell avidity for A24/WT1235 when paired with chain-centric TAK1β

As shown above, the TCRx 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 TCRx 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 TCRx genes were similarly reconstituted on the cell surface. Note that TCRx Cl. T4 paired with TAK1β was not stained with A24/WT1235 tetramer, because this transfectant was cross-reactive for B57 but not A24/WT1235 (Figs. 4A and 5A; 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 TCRx 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 TCRx transfectants individually expressing various A24/WT1235 TCRx 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 TCRx chains with structural and functional avidities over a broad range.
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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" motif using the ScanProsite tool (45, 46). We synthesized eleven human-derived peptides homologous to A24/WT1235 as shown in Supplementary Table S1. Jurkat 76/CDB TCN 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

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</tr>
<tr>
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<td>36’/D7’-04</td>
<td>CAV4G4P4R4D4K4IF</td>
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<td>CAV4T4G4T4S4YG4KLF</td>
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</tr>
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<td>CAV4E4L4I4Q4A4G4K4Q4FLF</td>
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<tr>
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<td>38-2/DV8’-01</td>
<td>CAV4R4S4R4N4G4S4G4N4KLTF</td>
<td>53’-01</td>
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NOTE: A24/WT1235 reactivity and B57 alloreactivity of 45 Jurkat 76/CDB TCR transfectants were measured using A24-aAPCs loaded with or without 1 μg/ml A24/WT1235 peptide and B57-aAPCs as stimulators in IL2 ELISPOT assays. All the experiments were performed in triplicate, and the mean number of IL2 SFUs per 4.0 x 10^4 cells was calculated. ++, >500 SFU/4.0 x 10^4 cells; +, 100–400 SFU/4.0 x 10^4 cells; –, <10 SFU/4.0 x 10^4 cells. All the transfectants produced <5 SFU per 4.0 x 10^4 cells when stimulated with HLA-null aAPCs (data not shown).

Abbreviation: SFU, spot-forming units.
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 Ya62 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 peptide and/or B57 molecules when paired with TAK1β were compared (left). The 21 TCR chains were divided into two groups, 8 TRAV36 TCR (center) and 13 non-TRAV36 TCRs genes (right). Each dot represents one donor. Pearson correlation coefficients were calculated; r and P values are shown.

Collectively, our results suggest that, although newly cloned A24/WT1235 reactivity and B57 alloreactivity of 21 TCR chains (Table 1) that recognize naturally processed and presented A24/WT1235 peptide and/or B57 molecules when paired with TAK1α chain. A, cloned TCR chains demonstrate A24/WT1235 reactivity and/or B57 alloreactivity when reconstituted with the TAK1α chain on Jurkat 76/CD8 cells. The A24/WT1235 reactivity and B57 alloreactivity of all 45 Jurkat 76/CD8 TCR transfectants were measured using the indicated aAPCs as stimulators in IL2 ELISPOT assays (Table 1). The A24/WT1235 reactivity and B57 alloreactivity of three representative TCR 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/WT1235 reactivity and B57 alloreactivity of TRAV36 but not non-TRAV36 TCR genes correlated. The A24/WT1235 reactivity and B57 alloreactivity of 21 TCR chains (Table 1) that recognize naturally processed and presented A24/WT1235 peptide and/or B57 molecules when paired with TAK1α were compared (left). The 21 TCR chains were divided into two groups, 8 TRAV36 TCR (center) and 13 non-TRAV36 TCR genes (right). Each dot represents one donor. Pearson correlation coefficients were calculated; r and P values are shown.

Figure 4.

B57 alloreactivity is separable from A24/WT1235 reactivity by changing the TCRα chains paired with the TAK1β chain. A, cloned TCR chains demonstrate A24/WT1235 reactivity and/or B57 alloreactivity when reconstituted with the TAK1α chain on Jurkat 76/CD8 cells. The A24/WT1235 reactivity and B57 alloreactivity of all 45 Jurkat 76/CD8 TCR transfectants were measured using the indicated aAPCs as stimulators in IL2 ELISPOT assays (Table 1). The A24/WT1235 reactivity and B57 alloreactivity of three representative TCR 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/WT1235 reactivity and B57 alloreactivity of TRAV36 but not non-TRAV36 TCR genes correlated. The A24/WT1235 reactivity and B57 alloreactivity of 21 TCR chains (Table 1) that recognize naturally processed and presented A24/WT1235 peptide and/or B57 molecules when paired with TAK1α were compared (left). The 21 TCR chains were divided into two groups, 8 TRAV36 TCR (center) and 13 non-TRAV36 TCRs genes (right). Each dot represents one donor. Pearson correlation coefficients were calculated; r and P values are shown.
possessed well-correlated A24/WT1235 reactivity and B57 allor-activity when paired with TAK1β, they may recognize A24/ WT1235 and B57 with a similar footprint (Fig. 4B). In contrast, non-TRAV36 TCRα clonotypes, which possess either A24/WT1235 reactivity or B57 allorreactivity exclusively, may bind A24/WT1235 or B57 using different conformations. Crystallographic analysis of
These distinct TCRα chains paired with the TAK1β chain is warranted.

Multiple clonotypic TCRα counter-chains were able to recognize A24/WT1235 and/or B57 when paired with TAK1β, and these TCRs possessed a very wide range (>3 log orders) of structural and functional avidities for A24/WT1235. Of note, TCRα hemi-chains, such as Cl. A262 and Cl. T262, were able to recognize naturally processed and presented A24/WT1235 peptide when reconstituted

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 alanine. The reactivity of Jurkat 76/CD8 cells reconstituted with Cl. T262, Cl. A262, or Cl. T53 along with TAK1β is shown as % IL2 maximum secretion capacity as determined by IL2 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 < 0.01. 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 12 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. IL2 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.
with TAK1β on Jurkat 76/CD8 cells, suggesting that they possess sufficient affinity to recognize tumor cells in the presence of CD8 coreceptors (Table 1). When reconstituted along with TAK1β in the absence of CD8 molecules, however, none of the isolated TCRα chains were stained with A24/WT1235 tetramer or recognized naturally processed and presented A24/WT1235 peptide (data not shown). High-affinity HLA class I-restricted TCRs that can recognize endogenously processed and presented peptides in the absence of CD8 coengagement 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 TAK1β-transduced CD8+ T cells with aAPCs expressing mutated A24 that cannot associate with CD8 may enrich TAK1β+ A24/WT1235 CD8+ T cells with higher avidity and facilitate the cloning of high-affinity TCRα chains that do not require CD8 coengagement 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/WT1235 TCRs do not recognize A24+CD34+ hematopoietic cells (Fig. 6C). And yet, because 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/WT1235 TCRs containing TAK1β chain possessed potential cross-reactivity to peptides homologous to A24/WT1235, 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 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.

Disclosure of Potential Conflicts of Interest
S. Tanaka is an employee of Takara Bio, Inc. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: N. Hirano

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References


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