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Specific Roles of Each TCR Hemichain in Generating Functional Chain-Centric TCR

Munehide Nakatsugawa,* Yuki Yamashita,* Toshiki Ochi,* Shinya Tanaka,* Kenji Chamoto,* Tingxi Guo,* Marcus O. Butler,*‖ and Naoto Hirano*†,‡

TCR-α and β-chains cooperatively recognize peptide–MHC complexes. It has been shown that a “chain-centric” TCR hemichain can, by itself, dictate MHC-restricted Ag specificity without requiring major contributions from the paired TCR counterchain. Little is known, however, regarding the relative contributions and roles of chain-centric and its counter, non–chain-centric, hemichains in determining T cell avidity. We comprehensively analyzed a thymically unselected T cell repertoire generated by transducing the α-chain-centric HLA-A*02:01(A2)/MART127–35 TCRα, clone SIG35α, into A2-matched and unmatched post-thymic T cells. Regardless of their HLA-A2 positivity, a substantial subset of peripheral T cells transduced with SIG35α gained reactivity for A2/MART127–35. Although the generated A2/MART127–35-specific T cells used various TRBV genes, TRBV27 predominated with >102 highly diverse and unique clonotypic CDR3β sequences. T cells individually reconstituted with various A2/MART127–35 TRBV27 TCRβ genes along with SIG35α possessed a wide range (>2 log orders) of avidity. Approximately half possessed avidity higher than T cells expressing clone DMF5, a naturally occurring A2/MART127–35 TCR with one of the highest affinities. importantly, similar findings were recapitulated with other self-Ags. Our results indicate that, although a chain-centric TCR hemichain determines Ag specificity, the paired counterchain can regulate avidity over a broad range (>2 log orders) without compromising Ag specificity. TCR chain centricity can be exploited to generate a thymically unselected Ag-specific T cell repertoire, which can be used to isolate high-avidity antitumor T cells and their uniquely encoded TCRs rarely found in the periphery because of tolerance. The Journal of Immunology, 2015, 194: 000–000.

Conventional αβ TCRs, which recognize peptide–MHC (pMHC) complexes, are composed of TCR-α- and β-chains, which both possess three CDR loops. The variable TCR-α or β CDR1 and 2 regions are encoded within the germline Vα or β segment, and the hypervariable CDR3 region is determined by the junction of spliced Vαs or VDβs gene segment accompanied by random insertion and deletion of nucleotides (1). The heterogeneity in these six TCR-α- and β-chain CDR regions coordinate determinates the breadth of target Ags and the affinity of a given TCR. Thus, the TCR CDR sequence diversity defines a repertoire of T cells, whose mission is to recognize and target a large array of foreign Ags as adaptive lymphocytes. The repertoire of naïve T cells is vast, if not infinite, and contains millions of unique TCR structures resulting from CDR sequence diversity. In the face of such diversity, expansion out of this gigantic repertoire of clonotypic T cells with Ag specificity and defined affinity was believed to be a largely stochastic and random process that results in a highly individualized response to an Ag.

However, accumulating evidence suggests that T cell responses exist where multiple individuals generate T cells with identical or near-identical TCRs in response to the same antigenic epitope. These shared or public TCRs have been observed to occur in many types of immune responses in multiple species across many facets of immunology including infectious diseases, malignancy, autoimmunity, and allergy (2, 3). It is believed that public TCRs result from a mixture of recombinatorial bias in the thymus and Ag-driven selection in the periphery. Public TCR-α- or β-chains can promiscuously pair with multiple clonotypic counterchains with various CDR3 sequences while preserving Ag specificity. For example, public clonotypic HLA-B*07:02 (B7)-restricted HSV-2 VP2249–57-specific TRAV1-1 TCR-α chains form functional heterodimers with TRBV5-1, 6-1, 9, and 12-3 TCR-β chains (4). In this example, the TCR-α chain appears dominant and contributes more to the overall strength of the TCR–pMHC interaction compared with paired TCR-β chains. In contrast, CD8+ T cell responses to an HLA-B7–restricted pp65265–275 epitope of human CMV was highly biased and frequently dominated by a public TRBV4-3 TCR-β chain (5). The presence of these public TCR hemichains that form Ag-specific heterodimers in conjunction with multiple clonotypic TCR counterchains suggests that either TCR-α- or β-chain can play a dominant role in binding pMHC complexes requiring minimal contributions from the counterchain.

The online version of this article contains supplemental material.

Abbreviations used in this article: aAPC, artificial APC; MART, melanocyte differentiation Ag; mut-aAPC, aAPC expressing mutated HLA-A2; pMHC, peptide–MHC; siMART1, small interfering RNA against MART1; wt-aAPC, aAPC expressing wild-type HLA-A2.

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Defining the relative contributions of TCRα- or β-chain in pMHC binding has been a topic of great interest. According to crystallographic studies, either TCRα or β hemichain can be dominant depending on the particular target pMHC complexes that are recognized (6, 7). The existence of dominant TCR hemichains has also been demonstrated using other approaches. Yokosuka et al. (8) reported that, when coexpressed with H-2Dβ-restricted HIVgp1601515–1592–specific TRAV16N/J32 TCRα-chain, clone RT1, one third of TRBV13-3 TCRβ-chains randomly chosen from naive mouse T cells were able to generate Ag-specific TCRβ dimers. Interestingly, Jβ usage affected the functional avidity of reconstituted TCRs. Using mice transgenic for the Dβ-restricted H-Y 738–746–specific TCR (16), Jβ held true with other self-Ags in addition to A2/MART1.

The primary CDR3 sequences span a range of avidities spanning 2 log orders that are solely dependent on the particular target pMHC complexes that are recognized using other approaches. Yokosuka et al. (8) found that this β-chain was able to pair with multiple TCRα-chains with various CDR3α sequences and that the TCR structure correlated with T cell avidity.

MART1, a melanocyte differentiation Ag, was identified as a target of HLA-A2–restricted CTLs isolated from patients with malignant melanoma (10, 11). Because MART1 is expressed by the majority of melanoma tumors but not by normal tissues except for normal melanocytes, a number of immunotherapy clinical trials have used MART1 as a target (12–20). It is well-known that the frequency of precursor CTLs specific for A2/MART127–35 (hereafter A2/MART1) is unusually high in HLA-A2+ healthy individuals (21, 22). TCR sequencing analysis of A2/MART1 CD8+ T cell clones isolated from tumor-infiltrating lymphocytes and PBLs demonstrated a striking bias in the usage of TRAV1-22 across different individuals (23, 24). Cole et al. suggested this bias could be because of the interaction between the TRAV1-22 CDR1α loop and the peptide, describing it as “innate-like” recognition of the pMHC complex (25). Both A2/MART1 TCR, clone MEL5, and A2/HTLV-1 TAX12–19 TCR, clone A6, bear TRAV1-22 TCRα-chains, but their CDR3α sequences are different: their TCRβ-chains use different TRBV genes and encode distinct CDR3β sequences. Interestingly, crystallographic studies revealed that MEL5 and A6 align in nearly identical positions and orientations over the cognate pMHC complex (25, 26). Based on this, it was suggested that the Vα subfamily, that is, CDR1/2 regions, of TRAV1-22 TCRα-chains play a dominant role in TCR: A2 docking with minimal contributions from heterogeneous TCRβ-chains, allowing TRAV1-22 TCRα-chains to bind A2–peptide complexes in an “α-centric” manner (25, 27, 28).

These studies suggest that a dominant TCR hemichain or TCR hemichain with chain centricity alone can largely dictate its MHC-restricted Ag specificity. However, virtually all studies analyzed peripheral T cells, which have undergone thymic selection, resulting in the substantial depletion of a subset of Ag-specific T cell precursors, especially those with high avidity. Accordingly, these studies may have underestimated the magnitude of heterogeneity and avidity of T cells that express a dominant TCR hemichain. Therefore, it remains to be determined, in the absence of constraints by thymic selection, how permissive a dominant TCR can be in selecting TCR counterchains while preserving Ag specificity, and how broad the range of TCR avidity can be for the cognate Ag complex.

To address these questions, we generated thymically unselected A2/MART1 TCR repertoires by transducing a public A2/MART1 TCRα-chain into human peripheral T cells from HLA-A2+ and -A2− donors. By using an artificial APC (aAPC)–based system, which can deliver a controlled level of T cell stimulation (29), we isolated highly polyclonal A2/MART1 T cells from these de novo A2/MART1 T cell repertoires and cloned their TCRβ-chains. T cells reconstituted with a single public A2/MART1 TCRα-chain along with various clonotypic TCRβ-chains possessed a wide range of avidities spanning 2 log orders that are solely dependent on the primary CDR3β structures. Importantly, similar findings held true with other self-Ags in addition to A2/MART1.

**Materials and Methods**

**Cells**

Peripheral blood samples were obtained from healthy donors after Institutional Review Board approval. All donors were identified to be positive or negative for HLA-A*02:01 (A2) by high-resolution HLA DNA typing (American Red Cross). Mononuclear cells were obtained by density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare). K562 is an erythroleukemic cell line used for HLA expression. T2 is an HLA-A2– T cell leukemia cell hybrid line. SupT1 is a a–TCRβγ- pre-T leukemia cell line. Jurkat 76 is a T cell leukaemic cell line lacking TCR and CD8 expression (a gift from Dr. M. Heemskerk, Leiden University Medical Center, Leiden, the Netherlands) (30). A375 (A2+ MART1+), Malme-3M (A2+ MART1−), SK-MEL-37 (A2+ MART1−), Me275 (A2+ NY-ESO-1−), SK-MEL-28 (A2+ MART1− NY-ESO-1−), and SK-MEL-21 (A2+ NY-ESO-1+) are melanoma cell lines. All cell lines were maintained in RPMI 1640 supplemented with 10% FCS and gentamicin (Invitrogen) as reported previously (20, 30–34). Melanoma cell lines were grown in DMEM medium supplemented with 10% FCS and gentamicin.

**eDNAs**

Codon-optimized A2/MART1 TCR gene (clone SIG35a) was produced by Life Technologies (Burlingame, CA) according to the published sequence (23, 24). Except for DMF5β, 1G4α, 1G4β, and 1G4Lyα genes, each TCR- or β-chain gene of interest was fused with ΔNGFR gene via an optimized intervening sequence consisting of a furin cleavage site, an SGSG spacer sequence, and an F2A sequence (35). Mutagenesis was conducted using standard molecular biology techniques. A2/MART1 TCR (clone DMF5) and A2/NY-ESO-1157–165 (hereafter named A2/NY-ESO-1) TCR (clone 1G4) genes were kindly provided by Dr. S. Rosenberg (National Institutes of Health/National Cancer Institute, Bethesda, MD). To clone TCR TRBV27 genes, we performed RT-PCR using TRBV27-specific primer, 5′-TRBV27 (5′- ATCGTCGACCACTGTGGCGGCCGCTCGAGCTAGCCTCTGGAATCCTTTCTCTTGACGTACGGCCCCCAAGCTCTTGGC-3′), and β C region–specific reverse primers, 3′-Cβ1 (5′- ATCTGGTGACACCTTGCTGGGGCCGGCCGCTGAGTTCCAGGGCTGCCTTCAGAAATCC-3′) or 3′-Cβ2 (5′- GACCACTGTGTCGTGGCGCCGCTGAGTTCCAGGGCTGCCTTCAGAAATCC-3′). Full-length MART1 and NY-ESO-1 eDNAs were cloned from melanoma cell lines into pMX vector. Sequencing was performed at the Centre for Applied Genomics, The Hospital for Sick Children (Toronto, ON). TCRα and β gene names in are in accordance with International ImMunoGeneTics Information System unique gene nomenclatures (http://www.imgt.org).

**Peptides**

Peptides used were A2-restricted wild-type MART127–35 (a2AAGICIGILTV3), helioreticulin NY-ESO-1157–165 (157SLMLWITQ165), and HIV pol476–484 (2A/HIV) (476GKPEPVEQ484) peptides. Synthetic peptides were obtained from ProImmune. A375 (A2+ NY-ESO-1+) or A2/NY-ESO-1 pre–TCR a chain in pMHC complexes in an “α-centric” manner (25, 27, 28).

These studies suggest that a dominant TCR hemichain or TCR hemichain with chain centricity alone can largely dictate its MHC-restricted Ag specificity. However, virtually all studies analyzed peripheral T cells, which have undergone thymic selection, resulting in the substantial depletion of a subset of Ag-specific T cell precursors, especially those with high avidity. Accordingly, these studies may have underestimated the magnitude of heterogeneity and avidity of T cells that express a dominant TCR hemichain. Therefore, it remains to be determined, in the absence of constraints by thymic selection, how permissive a dominant TCR can be in selecting TCR counterchains while preserving Ag specificity, and how broad the range of TCR avidity can be for the cognate Ag complex.

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**Transfectants**

SupT1 cells reconstituted with TCRs were purified using CD3 Microbeads (Miltenyi Biotec) according to the manufacturer’s instruction. Jurkat 76 was transduced with CD8α and CD8β eDNAs to generate Jurkat 76/CD8αβ as reported previously (37). Jurkat 76 or Jurkat 76/CD8αβ transfectants were further transduced with individual TCRγ genes along with SIG35a, and the transfectants were purified using CD3 Microbeads. K562-based aAPCs expressing wild-type HLA-A2 (wt-aAPC) and mutated HLA-A2 (mut-aAPC) in conjunction with CD80 and CD83 were reported elsewhere (37). Mutated HLA-A2 molecules bear two amino acid substitutions at positions 227 and 228 that abrogate the interaction with A2 (38). Mut-aAPC was engineered to constitutively express cDNA to generate T75 expansion (37). 293GFP-derived retrovirus supernatants were used to introduce TCR genes into SupT1 as reported previously (37). PG13-derived retrovirus supernatants were used to transduce TCR genes into Jurkat 76, Jurkat 76/CD8αβ, and human primary T cells. TransIT293 (Mirus Bio) was used to transfect TCR genes into packaging cell lines. A retroviral vector encoding ΔNGFR alone was used as a control vector. MART1 A275 was retrovirally transduced with full-length MART1 cDNA to generate T75 M1 T1. Similarly, NY-ESO-1 SK-MEL-21 and SK-MEL-28 were infected with retrovirus encoding full-length NY-ESO-1 cDNA to produce SK-MEL-21/NY-ESO-1 and SK-MEL-28/NY-ESO-1, respectively. HLA-A2−
SK-MEL-28 was retrovirally transduced with wild-type HLA-A2 to generate SK-MEL-28/A2. To knockdown the MART1 gene, we retrovirally infected target cells with small interfering RNAs against MART1 (siMART1) as reported previously (39). The target sequences of siMART1 were as follows: 5'-GAGAAGATGCTCACCTCATC-3', 5'-CAGTTTACCACCCAGGCTTGAA-3', 5'-GGCAGTTAATGGTGTTAACA-3', and 5'-AAGAGCAGAAA-TGGGATACAGAGC-3'. Malme-3M was transduced with the siMART1 using retrovirus system to generate Malme-3M/siMART1 with suppressed MART1 expression. 293GPG-derived retrovirus supernatants were used for retroviral transduction as reported previously (32, 37). The expression of MART1 and NY-ESO-1 in the transduced cells was evaluated by Western blot analysis with anti-MART1 (clone E978; Santa Cruz Biotechnology), respectively. HLA-A2 expression in the transduced cells was analyzed by flow cytometry after staining with anti–HLA-A2 (clone BB7.2; Biolegend) as reported previously (32).

Expansion of TCR gene-modified CD8+ T cells in an HLA-A2-restricted peptide-specific manner

Peptide-specific CD8+ T cells were expanded using an aAPC as described previously (31, 32, 40–42). PBMCs were isolated from healthy volunteers and stimulated with 50 ng/ml anti-CD3 mAb (clone OKT3) in the presence of 100 IU/ml human IL-2 (Novartis) 3 d before or on the day of restimulation. A2/HIV pol476–484 peptide was used as a peptide for 6 h at room temperature. The aAPC was then irradiated at 200 Gy, and stimulated with 50 ng/ml anti-CD3 mAb. T cells were retrovirally transduced with TCR genes by centrifuging 1 h at 1000 × g at 32˚C. After transduction, CD8+ T cells were purified and plated at 2 × 106 cells/well in RPMI 1640 supplemented with 10% human AB serum. The stimulator wt-aAPC or mut-aAPC was pulsed with 10 ng/ml A2/HIV multimer along with anti-CD3 mAb. Data shown are gated on aAPC and stained with anti-CD3 (clone UCHT1; Biolegend) as reported previously (32).

Flow-cytometry analysis

Cell-surface molecules on transfectants were counterstained with PC5-conjugated anti-CD8 mAb (clone B9.11; Beckman Coulter), FITC-conjugated anti-NFGR (clone ME20.4; Biolegend), and FITC-conjugated anti-CD3 (clone UCHT1; Biolegend). Assessment of TCR β subfamily usage was performed using TCR β mAbs (Beta Mark, Coulter, CA) as reported previously (31). Stained cells were analyzed with flow cytometry (BD Biosciences), and data analysis was performed using FlowJo (Tree Star) as published previously (40–43).

HLA/peptide multimer staining

Biotinylated HLA-A2/peptide monomers were purchased from ProImmune, multimerized in-house using streptavidin-PE and streptavidin-allophycocyanin, and used to stain Ag-specific T cells as described previously (20, 37, 44, 45). A2/HIV multimer was always used as a control. Structural avidity was determined by staining with graded concentrations of A2/MART1 multimer.

Cytokine ELISPOT analysis

IL-2- and IFN-γ ELISPOT assays were conducted as described elsewhere (37, 43–45). In brief, PVDF plates (Millipore) were coated with capture mAb. T cells were incubated with 2 × 105 per well of T2 cells in the presence of wild-type A2/MART127–35 peptide for 20–24 h at 37˚C. Plates were washed and incubated with biotin-conjugated detection mAb. Functional avidity was tested using T2 cells pulsed with graded concentrations of wild-type A2/MART127–35 peptide as stimulators in ELISPOT assays as reported previously (37).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0e. To determine whether two groups were statistically different for a given variable, we performed analysis using Welch’s t test (two-sided). The p values < 0.05 were considered significant.

Results

When paired with the endogenous irrelevant SupT1 TCRβ-chain, SIG35α, but not DMF5α, recognizes A2/MART1

The A2/MART1 TCRα gene, clone SIG35α (hereafter called SIG35α), uses TRAV12-2/J35. Although this TRAV-usage does not match with the previously described public A2/MART1 TCR, TRAV12-2/J34 or J45, SIG35α has been repeatedly isolated from A2/MART1 CTLs by many groups including us (3, 20, 23, 24, 46). SIG35α has been shown to pair with TRBV5-1 and TRBV27 TCRβ-chains with diverse CDR3β sequences, suggesting that recognition of

### Table I. CDR3 sequences of TCRβ-chains paired with SIG35α in A2/MART1 T cells

<table>
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<th>CDR3β</th>
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<td>24</td>
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</tr>
<tr>
<td>5-1</td>
<td>CASSLGGSSEQPF</td>
<td>2-1</td>
<td>24</td>
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</table>

**FIGURE 1.** SIG35α, but not DMF5α-expressing SupT1 cells are stained by A2/MART1 multimers when paired with the endogenous irrelevant TCRβ-chain of SupT1 cells. The human TCRαβ pre-T cell leukemia cell line, SupT1, was transduced with five different clonotypic A2/MART1-specific TCRα-chains: SIG35α/ΔNGFR, DMF5α/ΔNGFR, SIG35αN/ΔNGFR, or DMF5αS/ΔNGFR, or TCRβ-chains, DMF5αβ/ΔNGFR. SIG35αN is a SIG35α-derived mutant encoding Asn instead of Ser at the V-J junction. DMF5α is a DMF5α-derived mutant coding for Ser instead of Asn at the V-J junction. All TCRα genes were fused with ΔNGFR′ gene via an optimized intervening sequence consisting of a furin cleavage site, an SGSG spacer sequence, and an F2A sequence (35). ΔNGFR alone was used as a control. The transduction efficiency of SupT1 transfectants was ~90% as determined by the percentage of ΔNGFR+ cells (data not shown). All SupT1 transfectants were stained with A2/MART1 or A2/HIV multimer along with anti-CD3 mAb. Data shown are gated on ΔNGFR+ cells and are representative of two independent experiments.
FIGURE 2. Both HLA-A2+ and -A2− peripheral T cells can recognize A2/MART1 when transduced with chain-centric SIG35α. (A) Both HLA-A2+ and -A2− peripheral T cells become A2/MART1-reactive upon transduction of chain-centric SIG35α. Peripheral CD8+ T cells freshly isolated from two HLA-A2+ donors (donors 1 and 2) and two A2− donors (donors 3 and 4) were retrovirally transduced with ΔNGFR or SIG35α/ΔNGFR and stained with A2/MART1 multimer or A2/HIV multimer in conjunction with anti-CD8 mAb and anti-NGFR mAb. Data shown are gated on ΔNGFR+ cells. Data of donors 1 and 3 are representative of three independent experiments, and data of donors 2 and 4 are representative of two independent experiments. (B) SIG35α-transduced A2/MART1 CD8+ T cells expand in an A2/MART1-specific manner. A2+ and A2− CD8+ T cells transduced with SIG35α/ΔNGFR were stimulated with wt- or mut-aAPCs pulsed with wild-type A2/MART1 peptide once a week. Between stimulations, the T cells were supplemented with IL-2 (10 IU/ml) and IL-15 (10 ng/ml) every 3 d. Data depict A2/MART1 multimer staining performed after the first and second (Figure legend continues)
A2/MART1 by SIG35α-containing TCRs is α-chain centric (Table I). SupT1 is a human pre–T cell leukemia cell line, which expresses pre-TCRα and TRBV9/J2-1 TCRβ-chains but not a mature TCRα-chain (47). This suggests that the SupT1 was derived from T cells, which had yet to experience HLA-restricted selection in the thymus. When transduced with SIG35α, SupT1 cells were successively stimulated using wt-aAPC and the second and third stimulations using mut-aAPC. Data shown are gated on ΔNGFR+ cells. (B) A significant proportion of TRBV27 TCRβ-chains in the periphery can recognize A2/MART1 when paired with SIG35α. The percentage of A2/MART1 multimer+ cells in CD8+ TRBV27+ T cells transduced with SIG35α/ΔNGFR gene is shown.

A2/MART1 by SIG35α predominantly pairs with TRBV27 TCRβ-chains to recognize A2/MART1. (A) SIG35α/ΔNGFR-transduced peripheral CD8+ T cells from two HLA-A2+ and two A2- donors were stained with A2/MART1 multimer, mAbs for TCR Vβ subtypes, and anti-CD8 mAb. The percentage of A2/MART1 multimer+ T cells expressing each subtype is shown. Data shown are gated on ΔNGFR+ cells. (B) A significant proportion of TRBV27 TCRβ-chains in the periphery can recognize A2/MART1 when paired with SIG35α. The percentage of A2/MART1 multimer+ cells in CD8+ TRBV27+ T cells transduced with SIG35α/ΔNGFR gene is shown.
cessfully stained by A2/MART1 multimer but not control A2/HIV multimer (Fig. 1, left). In contrast, SupT1 cells transfected with the TCR α gene from the high-affinity A2/MART1 TCR, clone DMF5 (called DMF5 hereafter), which harbors TRAV12-2/J23, was not stained by A2/MART1 multimer (48). Surface CD3 expression on both transfectants was similarly upregulated, confirming the successful transduction and surface expression of both TCR α genes (Fig. 1, left). Supratransduction of DMF5 into SupT1 transduced with DMF5 rendered the transfectant positive for A2/MART1 multimer staining, further confirming the successful transduction of DMF5. These results indicate that, compared with DMF5, SIG35 α plays a dominant role in the recognition of A2/MART1 and requires minor contributions from TCR β-chains to determine its A2-restricted MART1 27–35 epitope specificity.

The CDR3α regions of SIG35 α and DMF5 α encode CAVSIGFGNVL and CAVNGFGGKLLIF, respectively. SIG35 α, but not DMF5 α, harbors a flexible amino acid, Ser at the V-J junction underlined. When SIG35αN, which is a SIG35α-derived mutant encoding Asn in lieu of Ser, was transduced into SupT1 cells, positive A2/MART1 multimer staining was largely lost, suggesting that the Ser residue was critical for the chain centricity of SIG35 α (Fig. 1, right). The DMF5α mutant, DMF5αS, which carries a Ser residue instead of Asn at the V-J junction, was not able to acquire stronger chain centricity compared with parental DMF5 α. This indicates that the mere existence of a flexible amino acid, Ser, at the V-J junction is not sufficient to confer chain centricity to A2/MART1 TCR α genes.

Both HLA-A2 + and -A2 - peripheral T cells recognize A2/ MART1 when transduced with chain-centric SIG35 α

Peripheral T cells from four donors, two each for HLA-A2 + and -A2 - individuals, were transduced with SIG35 α alone and stained by A2/MART1 multimer (Fig. 2A). To distinguish A2/MART1 T cells derived from untransduced and transduced T cells, we fused the SIG35 α gene to the NGFR gene by the F2A sequence as in Fig. 1. The overall transduction efficiency of peripheral T cells was ∼50–85% as determined by the percentage of NGFR + cells (Supplemental Fig. 1A). NGFR and A2/MART1 multimer double-positive cells were detectable in all donors tested regardless of their HLA-A2 positivity. Previously, we reported

Table II. Sequencing results of TCR TRBV27 chains isolated from A2/MART1 multimer+ CD8 + T cells

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<th>Unique Clonotypes, n</th>
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FIGURE 5. The structural and functional avidity range of A2/MART1 TCRs consisting of SIG35α is very broad and further enhanced by the presence of CD8. Jurkat 76 cells, which lack the expression of CD8αβ and endogenous TCRs, were retrovirally transduced with CD8αβ to produce Jurkat 76/CD8αβ. Jurkat 76 or Jurkat 76/CD8αβ cells were individually transduced with 11 distinct TRBV27 TCRβ-chains along with SIG35α or with DMF5αβ-chains. (A) A2/MART1 TCRs reconstituted on Jurkat 76 or Jurkat 76/CD8αβ transfectants were differentially stained by A2/MART1 multimer. All Jurkat 76 or Jurkat 76/CD8αβ transfectants were stained with 2 μg/ml A2/MART1 or A2/HIV multimer along with anti-CD3 mAb (top) or anti-CD8 mAb (bottom). Data for multimer staining of seven representative Jurkat 76 or Jurkat 76/CD8αβ transfectants are shown. Data for multimer staining of the remaining five transfectants are shown in Supplemental Fig. 3. (B) Reconstituted A2/MART1 TCRs are highly avid for A2/MART1 recognition. IL-2 ELISPOT assays were performed using seven representative Jurkat 76 or Jurkat 76/CD8αβ transfectants as responder cells. T2 cells pulsed with 10 μg/ml wild-type A2/MART1 or A2/HIV control peptide were used as stimulator cells (left panels). The A2+ MART1+ melanoma line, A375, and the A2+ MART1+ melanoma line, Malme-3M, were used as stimulator cells (right panels). All experiments were conducted in triplicate, and error bars show...
a series of human cell-based aAPCs, which can expand in vitro Ag-specific CD4+ and CD8+ T cells, and polyclonal CD3+ T cells (31, 32, 37, 40–45). When exogenously pulsed with wild-type A2/MART1 peptide, wt-aAPC or mut-aAPC successfully expanded A2/MART1 T cells (Fig. 2B). mut-aAPCs express mutated A2 molecules, which cannot engage CD8 coreceptors, so that they specifically expand a subset of A2-restricted, Ag-specific T cells with higher avidity (see later) (37). Importantly, when not exogenously pulsed with wild-type A2/MART1 peptide, both aAPCs failed to grow A2/MART1 T cells, suggesting that the observed expansion of A2/MART1 T cells is dependent on pulsed A2/MART1 peptide (data not shown). The T cells expressing SIG35α recognized A2+ target cells pulsed with wild-type A2/MART1, but not A2/HIV control peptide (Fig. 2C). Furthermore, they were capable of targeting unpulsed A2+ MART1+ Malme-3M tumor cells, but not A2+ MART1+ A375 tumor cells, suggesting that the SIG35α-transduced T cells possessed functional avidity sufficient to recognize endogenerously processed and presented A2/MART1 peptide.

Using primary T cells, we also confirmed the significance of the Ser residue located at the V-J junction of SIG35α for its chain centricity shown in Fig. 1. When the SIG35αN mutant was transduced into primary T cells, positivity for A2/MART1 multimer staining drastically decreased compared with when SIG35α was transduced (Fig. 2D). The DMF5αN mutant could not significantly upregulate the A2/MART1 multimer positivity over parental DMF5α, again suggesting that a Ser residue is insufficient for the observed α-chain centricity of SIG35α and only critical in the context of surrounding CDR3α sequences. As shown in Fig. 2D, although DMF5α-transduced CD8+ T cells were also stained by A2/MART1 multimer, the percentage of A2/MART1 multimer-positive T cells was substantially lower compared with SIG35α-transduced T cells. We further compared the A2/MART1 multimer positivity of the T cells transduced with SIG35α or DMF5α in three other donors before and after Ag-specific expansion (Supplemental Fig. 1B). The percentage of A2/MART1 multimer+ T cells in DMF5α-transduced T cells was consistently lower compared with SIG35α-transduced T cells, suggesting that, compared with DMF5α, SIG35α requires less contribution from TCRβ counterchains to recognize A2/MART1.

SIG35α predominantly pairs with TRBV27 TCRβ-chains to recognize A2/MART1

SIG35α expressed in A2/MART1+ T cells paired with various Vβ subfamilies in both A2+ and A2− donors (Fig. 3A). The percentage of the overall transduced T cells expressing each Vβ subfamily is shown in Supplemental Fig. 2. Intriguingly, SIG35α predominantly paired with TRBV27 TCRβ-chains to recognize A2/MART1 in all four donors tested, which was often observed with A2/MART1-specific T cells isolated from the periphery or tumor sites (23, 24, 31, 49–52). When SIG35α-transduced T cells were cotransduced with anti-TRBV27 mAb and A2/MART1 multimer, large fractions of up to 75% of peripheral TRBV27+ cells were double positive for SIG35α and A2/MART1 multimer (Fig. 3B). These results demonstrate that A2/MART1-specific TCRs can be generated by pairing SIG35α with a large portion of the unrelated TRBV27 TCRβ-chain repertoire. Furthermore, they also suggest that the TRBV27 CDR1 and CDR2β, but not CDR3β, regions primarily regulate the A2/MART1 specificity of SIG35α-containing TCRs.

To assess the CDR3β heterogeneity of TRBV27 TCRβ genes that paired with SIG35α for A2/MART1 reactivity, we molecularly cloned TRBV27 TCRβ genes from SIG35α+ A2/MART1 muti-

mer+ cells from one A2+ and one A2− donor. wt- and mut-aAPCs were used to expand SIG35α-transduced T cells with a broad range of avidity. Sequence analysis of the CDR3β region revealed that cloned TRBV27 TCRβ genes were highly heterogeneous (Fig. 4 and Table II). We isolated a total of 139 and 38 independent clonotypic TCRβ-chains from wt- and mut-aAPCs, respectively, with highly diverse CDR3β sequences and amino acid lengths. No clonotypic TCRβ gene was shared between the two donors (Table II). Only three TCRβ gene clones were shared between A2/MART1 T cells obtained after stimulation with wt- and mut-aAPCs in the A2− donor. No clonotypic TCRβ gene was shared by the T cells expanded by wt- and mut-aAPCs in the A2+ donor. Furthermore, except for Jβ1-3 and 1-6, all Jβ subfamilies were used (Fig. 4). These results demonstrate that SIG35α can pair with a highly diverse repertoire of TRBV27 TCRβ-chains to constitute a TCR specific for A2/MART1. This confirms that the TRBV27 CDR3β region does not play a significant role in determining the A2/MART1 specificity of SIG35α.

The avidity range of A2/MART1 TCRs consisting of SIG35α is very broad and further enhanced by the presence of CD8

To study the avidity range of SIG35α+ A2/MART1 T cells, we randomly selected five and six clonotypic TRBV27 TCRβ-chain genes cloned from SIG35α+ A2/MART1 T cells stimulated by wt- and mut-aAPCs, respectively. These 11 clonotypic TRBV27 TCRβ genes were individually reconstituted along with SIG35α on TCRβ chain-Jurkat 76 T cells in the presence or absence of CD8αβ (Fig. 5A and Supplemental Fig. 3). All 12 transfectants including the one expressing DMF5 demonstrated comparable surface CD3 expression, suggesting the equivalent expression level of transduced TCR genes (Fig. 5A, top panel). Except for those expressing Cl. 413 and 523, all transfectants were stained by A2/MART1 multimer in the absence of CD8αβ coreceptor expression, suggesting high structural avidity. When coexpressed with CD8αβ, these two clones became positive for the multimer albeit at a lower level (Fig. 5A, bottom panel). Coexpression of CD8αβ molecules also enhanced the A2/MART1 multimer staining of other transfectants with higher structural avidity.

Except for the one expressing Cl. 413, all Jurkat 76 transfectants tested recognized wild-type A2/MART1 peptide pulsed on target cells in the absence of CD8αβ coreceptor (Fig. 5B, left panels). Coexpression of CD8αβ enabled the Jurkat 76 cells expressing Cl. 413 to also be reactive (Fig. 5B, left panels). Jurkat 76 transfectants expressing Cl. 830 and 794 possessed higher functional avidity compared with other transfectants and recognized A2+ MART1+ Malme-3M tumor cells in the absence of CD8αβ (Fig. 5B, right panels). In our experimental condition, the functional avidity of DMF5-transduced CD8αβ Jurkat 76 cells was insufficient to recognize A2+ MART1+ Malme-3M tumor cells. However, when
CD8αβ molecules were expressed, all transfectants, except for the ones expressing Cl. 413 and 523, were able to recognize A2* MART1* Malme-3M tumor cells. The Cl. 413- and 523-expressing transfectants were unable to detect Malme-3M even in the presence of CD8αβ coexpression (Fig. 5B, right panels). To further demonstrate the specific recognition of A2* MART1* tumor cells by the reconstituted A2/MART1 TCRs, we generated A2* MART1* A375/MART1, A2* MART1* SK-MEL-28/A2, and A2* MART1*low Malme-3M/siMART1 cells (Supplemental Fig. 4A, 4B). Using these tumor cells as target cells, we demonstrated the A2/MART1-restricted recognition by the Jurkat 76/CD8αβ transfectants individually expressing the 11 distinct clonotypic A2/MART1 TCRs (Table III). Furthermore, we evaluated the cross-reactivity of these TCRs to MART1-related peptides derived from normal human proteins, which were reported by Dutoit et al. (53) (Table IV). The number of MART1-related peptides recognized by the 11 Jurkat 76/CD8αβ TCR transfectants (2.6 ± 1.0, mean ± SD) is not significantly higher compared with the 5 A2/MART1 CTL clones (2.4 ± 1.7, mean ± SD) reported by Dutoit’s group (53).

We then systematically evaluated and compared structural and functional avidities of all Jurkat 76 transfectants in the absence or presence of CD8αβ. As shown in Fig. 5C, these transfectants demonstrated a wide range of structural and functional avidities that can be generally augmented by the CD8αβ coexpression. Data for structural and functional avidities of all transfectants are summarized in Table V. These results demonstrate that A2/MART1 T cells expressing SIG35α can possess a broad spectrum of avidity (>2 log orders), which is regulated by the CDR3β sequence in the context of the CDR1/2β sequence of the TCR counterchains.

**TCR chain centricity is commonly observed with HLA-restricted antitumor TCRs**

We next investigated whether the observed TCR chain centricity is unique to A2/MART1, which is known to have an exceptionally high precursor frequency (21, 22), or ubiquitous to other HLA-restricted tumor-associated Ags. The TCR gene, clone 1G4, is specific for A2/NY-ESO-1 peptide (54, 55). The TCR 1G4α- and β-chains harbor TRAV21/J6 and TRBV6-5/J2-2, respectively. A TCRα-chain 1G4α variant, called clone 1G4LYα, derived from 1G4 carries two amino acid substitutions at the CDR3α region, which demonstrates a higher TCR affinity when paired with 1G4β (56). Peripheral T cells transfected with any of 1G4α, 1G4β, or 1G4LYα showed positive for A2/NY-ESO-1 mono- or dimmer staining (Fig. 6A, left panel). The expanded A2/NY-ESO-1–specific T cells expressing 1G4α were polyclonal but predominantly positive for TRBV6-5 (Fig. 6A, right panel). The T cells expressing 1G4α hemichain recognized A2* target cells pulsed with A2/NY-ESO-1 but not A2/HIV control peptide (Fig. 6B, left panel). Furthermore, they were capable of recognizing unpulsed A2* NY-ESO-1* Me275 tumor cells but not A2* NY-ESO-1* SK-MEL-21 or A2* NY-ESO-1* SK-MEL-28 tumor cells (Fig. 6B, right cells). To further confirm the specificity of the T cells expressing 1G4α hemichain, we ectopically transduced A2* SK-MEL-21 and A2* SK-MEL-28 with full-length NY-ESO-1 to generate A2* SK-MEL-21/NY-ESO-1 and A2* SK-MEL-28/NY-ESO-1 (Supplemental Fig. 4C). The 1G4α hemichain-transduced T cells recognized A2* NY-ESO-1* SK-MEL-21/NY-ESO-1, but not A2* NY-ESO-1* SK-MEL-28/NY-ESO-1 (Fig. 6B, right). These results strongly suggest that the 1G4α hemichain-transduced T cells possess functional avidity sufficient to recognize naturally processed and presented A2/NY-ESO-1 peptide in a specific manner. Taken all together, these results strongly suggest that the observed chain centricity of HLA-restricted self-Ag–specific TCRs...
We have shown that in the absence of constraints imposed by thymic selection, a single clonotypic TCR hemichain with chain centricity can, in conjunction with a heterogeneous repertoire of TCR counterchains, constitute functional self-Ag–specific TCRs with a broad range of affinity. A chain-centric TCR hemichain determines Ag specificity of T cells, whereas the paired TCR hemichain lacking chain centricity regulates avidity without perturbing Ag specificity.

When reconstituted on T cells, about half of clonotypic TCRs randomly selected from de novo–generated A2/MART1 TCR repertoires demonstrated higher avidity compared with DMF5, a naturally occurring A2/MART1 TCR with one of the highest affinities that has been used in TCR gene transfer clinical trials (57). These results demonstrate the following three steps may serve as a general strategy to isolate high-affinity Ag-specific TCRs by overcoming the hurdles of central and peripheral tolerance: 1) generation of a thymically unselected TCR repertoire by transducing an Ag-specific TCR hemichain regardless of its affinity into human peripheral T cells, 2) enrichment of high-avidity T cells by delivering a controlled magnitude of Ag-specific stimulation using our artificial aAPC-based system, and 3) cloning and selection of TCR counterchains.

To isolate high-affinity TCRs, several different strategies have been developed. Using phage and yeast display systems, many groups screened libraries of TCRs with random amino acid substitutions in any of six CDR regions (58–60). Other groups undertook a similar strategy using T cells as host cells for screening (56, 61–63). Computational structure-based methods for high-affinity TCR design and engineering have also been reported (64–67). In most of these studies, the libraries screened were composed of TCRs with fixed lengths of CDR loops in which amino acids were only substituted but not deleted or inserted. In this regard, our strategy is unique, because it can screen TCRs with various amino acid lengths of CDR3 regions as shown in Fig. 4. It is well-known that the mutations in CDR1/2 regions upregulate the overall TCR:pMHC affinity by mainly enhancing the affinity between TCR and MHC, but not TCR and pMHC (68, 69). Accordingly, high-affinity TCRs with CDR1/2 mutations often lead to the loss of peptide specificity (56, 70). In contrast, our strategy uses native sequences that do not incorporate any mutations to the CDR regions. And yet, there still remains a risk that high-affinity TCRs cloned using our strategy carry unwanted off-target toxicities. Any TCR used in TCR gene therapy must still be confirmed to lack unwanted on- and off-target toxicities (71–73).

The SIG35α TCR chain can recognize A2/MART1 when paired with TRBV5-1 in addition to TRBV27 (Table I). Although comprehensive analysis is awaited, preliminary experiments indeed confirmed that TRBV5-1 TCRβ-chains isolated from SIG35α-transduced A2/MART1 multimer+ T cells recognized A2/MART1 when reconstituted with SIG35α (data not shown). However, the majority of endogenous TCRβ-chains in SIG35α/A2/MART1 T cells bore TRBV27, but not TRBV5-1 (Fig. 3A). These results suggest that, to recognize A2/MART1, SIG35α requires a lower contribution from TRBV27 compared with TRBV5-1 TCRβ-chains. This is underpinnned by the fact that the CDR3 region of TRBV27 TCRβ-chains that recognized A2/MART1 in association with SIG35α was highly heterogeneous (Fig. 4).
As shown in Fig. 2D and Supplemental Fig. 1B, SIG35α, but not SIG35αN, DMF5α, or DMF5αS, demonstrated potent chain centricity when transduced into primary T cells. Importantly, however, peripheral T cells forced to express either of SIG35αN, DMF5α, or DMF5αS also showed substantially higher, albeit low, A2/MART1 multimer positivity (0.5–0.9%) compared with DNIFGFR-transduced control T cells (0.02–0.1%). Furthermore, the transduction of peripheral T cells with A2/NY-ESO-1 hemichains also rendered them positive for specific A2 multimer staining as shown in Fig. 6A. Taking into account that TCRs are intrinsically highly cross-reactive and that a single TCR can recognize more than a million different peptides (74), chain centricity is likely to be an inherent and shared attribute of many, if not all, TCRs. Although the number of donors studied in this study is limited, there were no apparent differences in the heterogeneity of A2/MART1 TCRβ-chains cloned from HLA-A2+ and -A2- donors (Fig. 4 and Table II). Furthermore, avidities of A2/MART1 TCRβ-chains isolated from A2+ and A2- donors did not seem to differ when reconstituted with SIG35α on human T cells (Table V).

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Table V. Functional and structural avidities of the A2/MART1 TCRs

<table>
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<tr>
<th>Clone</th>
<th>Donor</th>
<th>αAPC Used for Stimulation</th>
<th>TRBV</th>
<th>CDR3β</th>
<th>TRBJ</th>
<th>EC₅₀ (µM)</th>
<th>Functional Avidity with CD8</th>
<th>Structural Avidity with CD8</th>
</tr>
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<tbody>
<tr>
<td>Cl.794</td>
<td>3 (A2-)</td>
<td>mut-aAPC</td>
<td>27</td>
<td>CASSLLGDYGTFF</td>
<td>1-2</td>
<td>0.12</td>
<td>0.16</td>
<td>0.06</td>
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<td>Cl.830</td>
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<td>mut-aAPC</td>
<td>27</td>
<td>CASSLLGAYEQYFF</td>
<td>2-7</td>
<td>0.13</td>
<td>0.14</td>
<td>0.01</td>
</tr>
<tr>
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<td>1 (A2+)</td>
<td>mut-aAPC</td>
<td>27</td>
<td>CASSFLGMAAEFF</td>
<td>1-1</td>
<td>0.14</td>
<td>0.16</td>
<td>0.06</td>
</tr>
<tr>
<td>Cl.1606</td>
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<td>mut-aAPC</td>
<td>27</td>
<td>CASSLLGSYEQYFF</td>
<td>2-7</td>
<td>0.16</td>
<td>0.12</td>
<td>0.01</td>
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<tr>
<td>Cl.1574</td>
<td>1 (A2+)</td>
<td>mut-aAPC</td>
<td>27</td>
<td>CASSPWERITEXAFF</td>
<td>1-1</td>
<td>0.35</td>
<td>0.15</td>
<td>0.1</td>
</tr>
<tr>
<td>Cl.1593</td>
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<td>27</td>
<td>CASGNOPQOHF</td>
<td>1-5</td>
<td>0.44</td>
<td>0.14</td>
<td>0.01</td>
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<tr>
<td>DMF5α</td>
<td>6-4</td>
<td>mut-aAPC</td>
<td>6-4</td>
<td>CASSLSQPTEAFF</td>
<td>1-1</td>
<td>1.4</td>
<td>0.33</td>
<td>0.03</td>
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<tr>
<td>Cl.758</td>
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<td>wt-aAPC</td>
<td>27</td>
<td>CASSPLAGDGEAFF</td>
<td>2-2</td>
<td>1.6</td>
<td>0.46</td>
<td>2.7</td>
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<tr>
<td>Cl.1086</td>
<td>3 (A2-)</td>
<td>wt-aAPC</td>
<td>27</td>
<td>CASSLHPGQYTF</td>
<td>1-2</td>
<td>2.4</td>
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<td>27</td>
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<td>27</td>
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<td>1-4</td>
<td>n.m.</td>
<td>10</td>
<td>n.m.</td>
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*Functional avidity, expressed as EC₅₀ in µM, was defined as the concentration of peptide required to achieve 50% of maximal response.

aStructural avidity, expressed as EC₅₀ in µg/ml, was defined as the concentration of A2/MART1 multimer required to achieve half-maximal multimer staining.

bDMF5 is a high-affinity A2/MART1 TCR described by Johnson et al. (48, 57).
n.m., not measurable.

As shown in Fig. 2D and Supplemental Fig. 1B, SIG35α, but not SIG35αN, DMF5α, or DMF5αS, demonstrated potent chain centricity when transduced into primary T cells. Importantly, however, peripheral T cells forced to express either of SIG35αN, DMF5α, or DMF5αS also showed substantially higher, albeit low, A2/MART1 multimer positivity (0.5–0.9%) compared with DNIFGFR-transduced control T cells (0.02–0.1%). Furthermore, the transduction of peripheral T cells with A2/NY-ESO-1 hemichains also rendered them positive for specific A2 multimer staining as shown in Fig. 6A. Taking into account that TCRs are intrinsically highly cross-reactive and that a single TCR can recognize more than a million different peptides (74), chain centricity is likely to be an inherent and shared attribute of many, if not all, TCRs. Although the number of donors studied in this study is limited, there were no apparent differences in the heterogeneity of A2/MART1 TCRβ-chains cloned from HLA-A2+ and -A2− donors (Fig. 4 and Table II). Furthermore, avidities of A2/MART1 TCRβ-chains isolated from A2+ and A2− donors did not seem to differ when reconstituted with SIG35α on human T cells (Table V). These results suggest that HLA-restricted thymic selection does
not affect TCR hemichain repertoires that can constitute functional TCRs in conjunction with a chain-centric TCR counterchain. Also, this raises the possibility that a TCR hemichain without chain centrality can constitute TCRs specific for various HLA-restricted Ags when paired with cognate Ag-specific chain-centric TCR counterchains. It has been recently noted that the overlap in the naïve CD8+ TCRβ sequence repertoires of any two of the individuals is ~7000-fold larger than predicted and seems to be independent of the degree of HLA matching (75). Importantly, these sequencing studies were performed at a population level, but not a single-cell level, and, therefore, did not consider pairings of clonotypic TCRα- and β-chains. Our results suggest that pairings of TCRα- and β-chains can be a critical determinant of TCR repertoire diversity, and that a different pairing can obviously make a de novo TCR repertoire and greatly enlarge its size.

Adoptive transfer of TCR gene-modified T cells is a feasible and promising treatment modality of cancer immunotherapy (15, 57, 76). When peripheral T cells are transduced with therapeutic TCRαβ genes, four different TCR chain pairings can be formed, including the therapeutic TCRαβ, the endogenous TCRαβ, and two mispaired TCRαβ dimers composed of the introduced TCRα or β with the endogenous TCRβ- or α-chains. These four potential TCRαβ dimers each compete for a fixed amount of endogenous CD3 complexes. Consequently, the density of the therapeutic TCR dimers on cell surface is reduced, leading to the decreased T cell avidity (77). Moreover, the mispaired TCRs may acquire unwanted specificity for unknown Ags, which can evoke harmful autotoxicities (78, 79). A number of different approaches have been developed to facilitate the matched pairing of the introduced TCR (80). The use of mouse instead of human TCR constant regions (81), the introduction of additional cytostate residues into TCR constant regions (82, 83), the usage of stabilized Vα/Vβ single-chain TCRs (84), and a knockdown of endogenous TCRs by zinc-finger nucleases (85) or small interfering RNAs (86) have been studied in vitro and in vivo. Although the transduction of both TCRαβ- and β-chains generates two types of mispaired TCRs, the transduction of TCR hemichain alone produces only one TCR mispairing. Accordingly, in theory, transducing a single TCR hemichain alone would reduce in half the issues associated with the transduction of TCR heterodimers. However, it is still mandatory to carefully monitor for possible unwanted harmful autotoxicities caused by the transduction of a TCR hemichain. In addition, it would still be necessary to knock down endogenous TCR hemichain of the same class as the introduced hemichain. It should be noted that our aAPC-based system to expand Ag-specific CD8+ T cells has been successfully translated into the clinic (29). Adoptive transfer of antitumor T cells generated in vitro using the system induced sustained clinical responses in patients with advanced cancer without any in vivo modulation such as cytokine administration or lymphodepletion (20). Clinical trials where patients are infused with antitumor T cells redirected by a chain-centric TCR hemichain and subsequently enriched by the aAPC-based system are warranted.

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Disclosures
S.T. is an employee of Takara Bio, Inc. The University Health Network has filed a provisional patent application related to this study on which N.H., M.N., and T.O. are named as inventors. The other authors have no financial conflicts of interest.

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