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Specific roles of each TCR hemichain in generating functional chain-centric TCR

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Abstract

T cell receptor (TCR)α and β chains cooperatively recognize peptide-MHC (pMHC) complexes. It has been shown that a 'chain-centric' TCR hemichain can, by itself, dictate MHC-restricted antigen 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)/MART1$_{27–35}$ 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/MART1$_{27–35}$. While the generated A2/MART1$_{27–35}$-specific T cells used various TRBV genes, TRBV27 predominated with >10^2 highly diverse and unique clonotypic CDR3β sequences. T cells individually reconstituted with various A2/MART1$_{27–35}$ 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/MART1$_{27–35}$ TCR with one of the highest affinities. Importantly, similar findings were recapitulated with other self-antigens. Our results indicate that, although a chain-centric TCR hemichain determines antigen specificity, the paired counterchain can regulate avidity over a broad range (>2 log orders) without compromising antigen specificity. TCR chain centricity can be exploited to generate a thymically unselected antigen-specific T cell repertoire,

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Author contributions

M.N. and N.H. designed the project. M.N., Y.Y., T.O., S.T., T.G., and K.C. performed the experimental work. M.O.B. provided human samples. M.N., M.O.B., and N.H. analyzed the results and wrote the manuscript.

Competing financial interests

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which can be used to isolate high avidity antitumor T cells and their uniquely encoded TCRs rarely found in the periphery due to tolerance.

Introduction

Conventional αβ T cell receptors (TCRs), which recognize peptide-MHC (pMHC) complexes, are comprised of TCRα and β chains, which both possess three complementarity-determining region (CDR) loops. The variable TCRα 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 VJα or VDJβ gene segment accompanied by random insertion and deletion of nucleotides (1). The heterogeneity in these 6 TCRα and β chain CDR regions coordinately determines the breadth of target antigens 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 antigens 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 antigen specificity and defined affinity was believed to be a largely stochastic and random process that results in a highly individualized response to an antigen.

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 antigen-driven selection in the periphery. Public TCRα or β chains can promiscuously pair with multiple clonotypic counterchains with various CDR3 sequences while preserving antigen specificity. For example, public clonotypic HLA-B*07:02 (B7)-restricted HSV-2 $VP22_{49–57}$-specific TRAV1-1 TCRα chain forms functional heterodimers with TRBV5-1, 6-1, 9, and 12-3 TCRβ chains (4). In this example, the TCRα chain appears dominant and contributes more to the overall strength of the TCR:pMHC interaction compared with paired TCRβ chains. In contrast, $CD8^+$ T cell responses to an HLA-B7-restricted $pp65_{265–275}$ epitope of human CMV was highly biased and frequently dominated by a public TRBV4-3 TCRβ chain (5). The presence of these public TCR hemichains which form antigen-specific heterodimers in conjunction with multiple clonotypic TCR counterchains suggests that either TCRα or β chain can play a dominant role in binding pMHC complexes requiring minimal contributions from the counterchain.

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 is recognized (6, 7). The existence of dominant TCR hemichains has also been demonstrated using other approaches. Yokosuka et al. reported that, when coexpressed with H-$2D^d$-restricted $HIVgp160_{315–329}$-specific TRAV16N/J32 TCRα chain, clone RT-1, one-third of TRBV13-3 TCRβ chains

randomly chosen from naive mouse T cells were able to generate antigen-specific TCRαβ dimers (8). Interestingly, Jβ usage affected the functional avidity of reconstituted TCRs. Using mice transgenic for the D^b-restricted H-$Y_{738–746}$-specific TCRβ chain, Bouneaud et al. found that this β chain was able to pair with multiple TCRα chains with various CDR3α sequences and that the TCRα structure correlated with T cell avidity (9).

MART1, a melanocyte differentiation antigen, was identified as a target of HLA-A2-restricted cytotoxic T lymphocytes (CTLs) isolated from patients with malignant melanoma (10, 11). Since 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 utilized MART1 as a target (12–20). It is well known that the frequency of precursor CTLs specific for A2/$MART1_{27–35}$ (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 peripheral blood lymphocytes demonstrated a striking bias in the usage of TRAV12-2 across different individuals (23, 24). Cole et al. suggested this bias could be due to the interaction between the TRAV12-2 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-I $TAX_{12–19}$ TCR, clone A6, bear TRAV12-2 TCRα chains but their CDR3α sequences are different: their TCRβ chains utilize 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 segments, i.e. CDR1/2 regions, of TRAV12-2 TCRα chains play a dominant role in TCR:A2 docking with minimal contributions from heterogeneous TCRβ chains, allowing TRAV12-2 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 antigen specificity. However, virtually all studies analyzed peripheral T cells, which have undergone thymic selection that results in the substantial depletion of a subset of antigen-specific T cell precursors, especially those with high avidity. Accordingly, these studies may have underestimated the magnitude of heterogeneity and avidity of T cells which 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 antigen specificity, and how broad the range of TCR avidity can be for the cognate antigen 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 utilizing an artificial antigen-presenting cell-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 avidities spanning 2 log orders that is solely dependent on the primary CDR3β structures. Importantly, similar findings held true with other self-antigens in addition to A2/MART1.

Materials and Methods

Cells

Peripheral blood samples were obtained from healthy donors following 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 defective for HLA expression. T2 is an HLA-A2 positive T cell leukemia/B-LCL hybrid cell line. SupT1 is a preTCRα^+/β^+ pre-T cell leukemia cell line. Jurkat 76 is a T cell leukemic cell line lacking TCR and CD8 expression (a gift from Dr. Heemskerk) (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 except for melanoma cell lines were cultured in RPMI1640 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.

cDNAs

Codon-optimized A2/MART1 TCR gene (clone SIG35α) was produced by Life Technologies (Burlingame, CA) according to the published sequence (23, 24). Except for DMF5β, 1G4α, 1G4β and 1G4LYα genes, each TCRα 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-$1_{157–165}$ (hereafter named A2/NY-ESO-1) TCR (clone 1G4) genes were kindly provided by Dr. Rosenberg (NIH/NCI, Bethesda, MD). To clone TCR TRBV27 genes, RT-PCR was performed using TRBV27-specific primer, 5′-TRBV27 (5′-ATCCCAGTGTGGTGGTACGGGAATTCTGCCATGGGCCCCCAGCTCCTTGGC-3′), and β constant region specific reverse primers, 3′-Cβ-1 (5′-ATCGTCGACCACTGTGCTGGCGGCCGCTCGAGTTCCAGGGCTGCCTTCAGAAATCC-3′) or 3′-Cβ-2 primer (5′-GACCACTGTGCTGGCGGCCGCTCGAGCTAGCCTCTGGAATCCTTTCTCTTGACCATTGC-3′). Full-length MART1 and NY-ESO-1 cDNAs were cloned from Malme-3M and Me275 cells by RT-PCR according to published sequences, respectively. cDNAs were cloned into pMX vector and utilized to transduce all cell lines and primary human T cells (36). Nucleotide sequencing was performed at the Centre for Applied Genomics, The Hospital for Sick Children (Toronto, Canada). TCRα and β gene allele names are in accordance with IMGT unique gene nomenclatures (http://www.imgt.org/).

Peptides

Peptides used were A2-restricted wild-type $MART1_{27–35}$ ($_{27}$AAGIGILTV$_{35}$), heteroclitic NY-ESO-$1_{157–165}$ ($_{157}$SLLMWITQV$_{165}$), and HIV $pol_{476–484}$ (A2/HIV) ($_{476}$ILKEPVHGV$_{484}$) peptides. Synthetic peptides were obtained from ProImmune. A2/HIV $pol_{476–484}$ peptide was always used as control peptide. Throughout the study, wild-type but

not heteroclitic A2/MART1 peptide was utilized for expansion and functional analysis of T cells.

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 cDNAs to generate Jurkat 76/CD8αβ as reported previously (37). Jurkat 76 or Jurkat 76/CD8αβ transfectants were further transduced with individual TCRβ genes along with SIG35α and the transfectants were purified using CD3 Microbeads. K562-based aAPCs expressing 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 which abrogate the interaction with A2 (38). Mut-aAPC was engineered to constitutively secrete IL-21 to enable T cell expansion (37). 293GPG-derived retrovirus supernatants were used to introduce TCR genes into SupT1 as reported previously (37). PG13-derived retrovirus supernatants were utilized 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 employed as a control vector. MART1-negative A375 was retrovirally transduced with full-length MART1 cDNA to generate A375/MART1. Similarly, NY-ESO-1-negative 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 negative SK-MEL-28 was retrovirally transduced with wild-type HLA-A2 to generate SK-MEL-28/A2. To knockdown the MART1 gene, target cells were retrovirally infected with small-interfering RNAs against MART1 (siMART1) as reported previously (39). The target sequences of siMART1 were as follows: 5′-GAGAAGATGCTCACTTCATCT-3′, 5′-CACTCTTACACCACGGCTGAA-3′, 5′-GGCACTCAATGTGCCTTAACA-3′ and 5′-AAGACGAAATGGATACAGAGC-3′. Malme-3M was transduced with the siMART1 using retrovirus system to generate Malme-3M/siMART1 with suppressed MART1 expression. 293GPG-derived retrovirus supernatants 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 A103; Santa Crus Biotechnology) and anti-NY-ESO-1 (clone E978; Santa Crus Biotechnology), respectively. HLA-A2 expression in SK-MEL-28/A2 cells was analyzed by flow cytometry following 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 days before transduction. Activated T cells were retrovirally transduced with TCR genes by centrifuging 1 hour at 1,000 g at 32°C. Following transduction, CD8$^+$ T cells were purified and plated at 2×10^6 cells/well in RPMI 1640 supplemented with 10% human AB serum. The stimulator wt-aAPC or mut-aAPC was pulsed with 10 μg/ml A2-restricted wild-type

MART1$_{27–35}$ or heteroclitic NY-ESO-1$_{157–165}$ peptide for 6 hours at room temperature. The aAPC was then irradiated at 200 Gy, washed, and added to the responder T cells at a responder to stimulator ratio of 20:1. Starting the next day, 10 IU/ml IL-2 (Novartis) and 10 ng/ml IL-15 (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. A2/HIV pol$_{476–484}$ peptide was used as a control.

Flow cytometry analysis

Cell surface molecules on transfectants were counterstained with PC5-conjugated anti-CD8 mAb (clone B9.11, Beckman Coulter), FITC-conjugated anti-NGFR (clone ME20.4; Biolegend), and FITC-conjugated anti-CD3 (clone UCHT1; Biolegend). Assessment of TCR Vβ subfamily usage was performed using TCR Vβ 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 (TreeStar) as published previously (40–43).

HLA/peptide multimer staining

Biotinylated HLA-A2/peptide monomers were purchased from ProImmune, multimerized in-house using SA-PE and SA-APC, and utilized to stain antigen-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). Briefly, PVDF plates (Millipore) were coated with capture mAb. T cells were incubated with 2×10^4 per well of T2 cells in the presence of wild-type A2/MART1$_{27–35}$ peptide for 20–24 hours 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/MART1$_{27–35}$ 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, analysis was performed using the Welch's *t* test (two-sided). *P* values of < 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) utilizes TRAV12-2/J35. Although this TRAV-J 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 A2/MART1 by SIG35α containing TCRs is α chain centric (Table I). SupT1 is a human pre-T cell leukemia cell line, which expresses preTCRα 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 successfully stained by A2/MART1 multimer but not control A2/HIV multimer (Fig. 1, left). In contrast, SupT1 cells transfected with the TCRα 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 TCRα genes (Fig. 1, left). Supra-transduction of DMF5β into SupT1 transduced with DMF5α rendered the tranfectant positive for A2/MART1 multimer staining, further confirming the successful transduction of DMF5α. These results indicate that, compared to DMF5α, SIG35α plays a dominant role in the recognition of A2/MART1 and requires minor contributions from TCRβ chains to determine its A2-restricted $MART1_{27–35}$ epitope specificity.

The CDR3α regions of SIG35α and DMF5α encode CAVSIGFGNVL and CAVNFGGGKLIF, respectively. SIG35α but not DMF5α harbors a flexible amino acid, Ser at the V-J junction as 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 SIG35α (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 to parental DMF5α. 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 TCRα genes.

Both HLA-$A2^+$ and $A2^-$ peripheral T cells recognize A2/MART1 when transduced with chain-centric SIG35α

Peripheral T cells from 4 donors, 2 each for HLA-$A2^+$ and $A2^-$ individuals, were transduced with SIG35α alone and stained by A2/MART1 multimer (Fig. 2A). To distinguish A2/MART1 T cells derived from untransduced and transduced T cells, the SIG35α gene was fused to the ΔNGFR gene by the F2A sequence as in Fig. 1. The overall transduction efficiency of peripheral T cells was approximately 50–85% as determined by the percentage of $\Delta NGFR^+$ cells (Supplementary Fig. 1A). ΔNGFR and A2/MART1 multimer double positive cells were detectable in all donors tested regardless of their HLA-A2 positivity. Previously, we reported a series of human cell-based artificial antigen-presenting cells (aAPCs), which can expand *in vitro* antigen-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, aAPCs expressing wild-type HLA-A2 (wt-aAPC) or mutated HLA-A2 (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 antigen-specific T cells with higher avidity (see below) (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 endogenously 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 to when SIG35α was transduced (Fig. 2). The DMF5αS 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 $multimer^+$ T cells was substantially lower compared to SIG35α-transduced T cells. We further compared the A2/MART1 multimer positivity of the T cells transduced with SIG35α or DMF5α in 3 other donors prior to and following antigen-specific expansion (Supplementary Fig. 1B). The percentage of A2/MART1 $multimer^+$ T cells in DMF5α-transduced T cells was consistently lower compared to SIG35α transduced T cells, suggesting that, compared to DMF5α, SIG35α requires less contribution from TCRβ counter-chains 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-positive and negative donors (Fig. 3A). The percentage of the overall transduced T cells expressing each Vβ subfamily is shown in Supplementary Fig. 2. Intriguingly, SIG35α predominantly paired with TRBV27 TCRβ chains to recognize A2/MART1 in all 4 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 costained 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 2β but not CDR3β region primarily regulate the A2/MART1 specificity of SIG35α containing TCRs.

TRBV27 TCRβ chains that can recognize A2/MART1 when paired with SIG35α are highly heterogeneous and unique

In order to assess the CDR3β heterogeneity of TRBV27 TCRβ genes which paired with SIG35α for A2/MART1 reactivity, TRBV27 TCRβ genes were molecularly cloned from $SIG35\alpha^+$ A2/MART1 multimer positive 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, 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 utilized (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 $SIG35\alpha^+$ A2/MART1 T cells, we randomly selected 5 and 6 clonotypic TRBV27 TCRβ chain genes cloned from $SIG35\alpha^+$ A2/MART1 T cells stimulated by wt- and mut-aAPCs, respectively. These 11 clonotypic TRBV27 TCRβ genes were individually reconstituted along with SIG35α on $TCR^{-/-}$ Jurkat 76 T cells in the presence or absence of CD8αβ (Fig. 5A, Supplementary 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). 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). 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). Coexpression of CD8αβ enabled the Jurkat 76 cells expressing Cl. 413 to also be reactive (Fig. 5B, left). 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). In our experimental condition, the functional avidity of DMF5-transduced $CD8\alpha\beta^-$ 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). 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 (Supplementary Fig. 4A, B). Using these tumor cells as target cells, we demonstrated the A2/MART1-restricted recognition by the Jurkat 76/CD8αβ transfectants individually expressing the eleven 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 to the 5 A2/MART1 CTL clones (2.4 ± 1.7, mean ± SD) reported by Dutoit's group (53).

We then systemically 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 which 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 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 antigens. 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 positivity for A2/NY-ESO-1 multimer staining (Fig. 6A, left). The expanded A2/NY-ESO-1-specific T cells expressing 1G4α were polyclonal but predominantly positive for TRBV6-5 (Fig. 6A, right). 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). Furthermore, they were capable of recognizing unpulsed $A2^+$ NY-ESO-1^+ Me275 tumor cells but not $A2^+$ NY-ESO-1^- SK-MEL-21 nor $A2^-$ NY-ESO-1^- SK-MEL-28 tumor cells (Fig. 6B, right). To further confirm the specificity of the T cells expressing 1G4α hemichain, A2-positive SK-MEL-21 and A2-negative SK-MEL-28 were ectopically transduced with full-length NY-ESO-1 to generate $A2^+$ SK-MEL-21/NY-ESO-1 and $A2^-$ SK-MEL-28/NY-ESO-1 (Supplementary 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-antigen-specific TCRs is a prevailing phenomenon, and can be exploited to efficiently isolate highly avid T cells and encoded TCRs specific for any HLA-restricted tumor-associated and pathogen-derived antigen.

Discussion

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 antigen-specific TCRs with a

broad range of affinity. A chain-centric TCR hemichain determines antigen-specificity of T cells, while the paired TCR hemichain lacking chain centricity regulates avidity without perturbing antigen-specificity.

When reconstituted on T cells, about one half of clonotypic TCRs randomly selected from *de novo* generated A2/MART1 TCR repertoires demonstrated higher avidity compared to 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 3 steps may serve as a general strategy to isolate high affinity antigen-specific TCRs by overcoming the hurdles of central and peripheral tolerance: 1) generation of a thymically unselected TCR repertoire by transducing an antigen-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 antigen-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. Utilizing phage and yeast display systems, many groups screened libraries of TCRs with random amino acid substitutions in any of 6 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 are comprised of TCRs with fixed lengths of CDR loops in which amino acids were only substituted but not deleted nor inserted. In this regard, our strategy is unique, since 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). While 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, in order to recognize A2/MART1, SIG35α requires a lower contribution from TRBV27 compared to TRBV5-1 TCRβ chains. This is underpinned 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 Supplementary Fig. 1B, SIG35α but not SIG35αN, DMF5α, nor 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 to ΔNGFR-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. Taken into account that TCRs are intrinsically highly crossreactive 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, 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 centricity can constitute TCRs specific for various HLA-restricted antigens when paired with cognate antigen-specific chain-centric TCR counterchains. It has been recently noted that the overlap in the naïve $CD8^+$ CDR3 sequence repertoires of any two of the individuals is approximately 7,000-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 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 comprised 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 antigens, which can evoke harmful autotoxicities (78, 79). To facilitate the matched pairing of the introduced TCR, a number of different approaches have been developed (80). The use of mouse instead of human TCR constant regions (81), the introduction of additional cysteine 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 (39), have been studied *in vitro* and *in vivo*. While the transduction of both TCRα 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 antigen-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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations in this paper

aAPC	artificial APC
pMHC	peptide/MHC
wt-aAPC	wild-type HLA-A2 aAPC
mut-aAPC	mutated HLA-A2 aAPC

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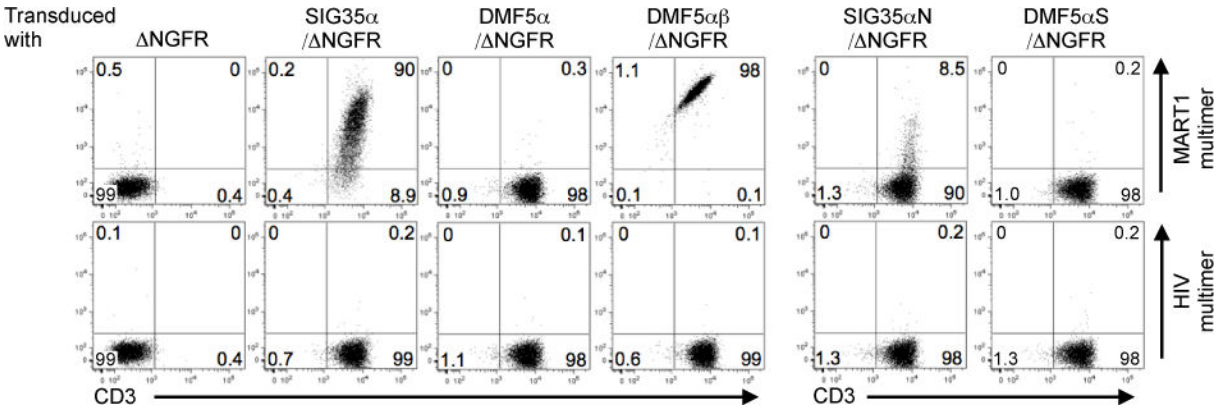

Fig. 1. SIG35α but not DMF5α expressing SupT1 cells are stained by A2/MART1 multimers when paired with the endogenous irrelevant TCRβ chain of SupT1 cells

The human TCRα^{-}/β^{+} pre-T cell leukemia cell line, SupT1 was transduced with 5 different clonotypic A2/MART1-specific TCRα chains, SIG35α/ΔNGFR, DMF5α/ΔNGFR, SIG35αN/ΔNGFR, or DMF5αS/ΔNGFR, or TCRαβ chains, DMF5αβ/ΔNGFR. SIG35αN is a SIG35α-derived mutant encoding Asn instead of Ser at the V-J junction. DMF5 is a high affinity A2/MART1 TCR (48). DMF5αS 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 employed as a control. The transduction efficiency of SupT1 transfectants was approximately 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 a representative of two independent experiments.

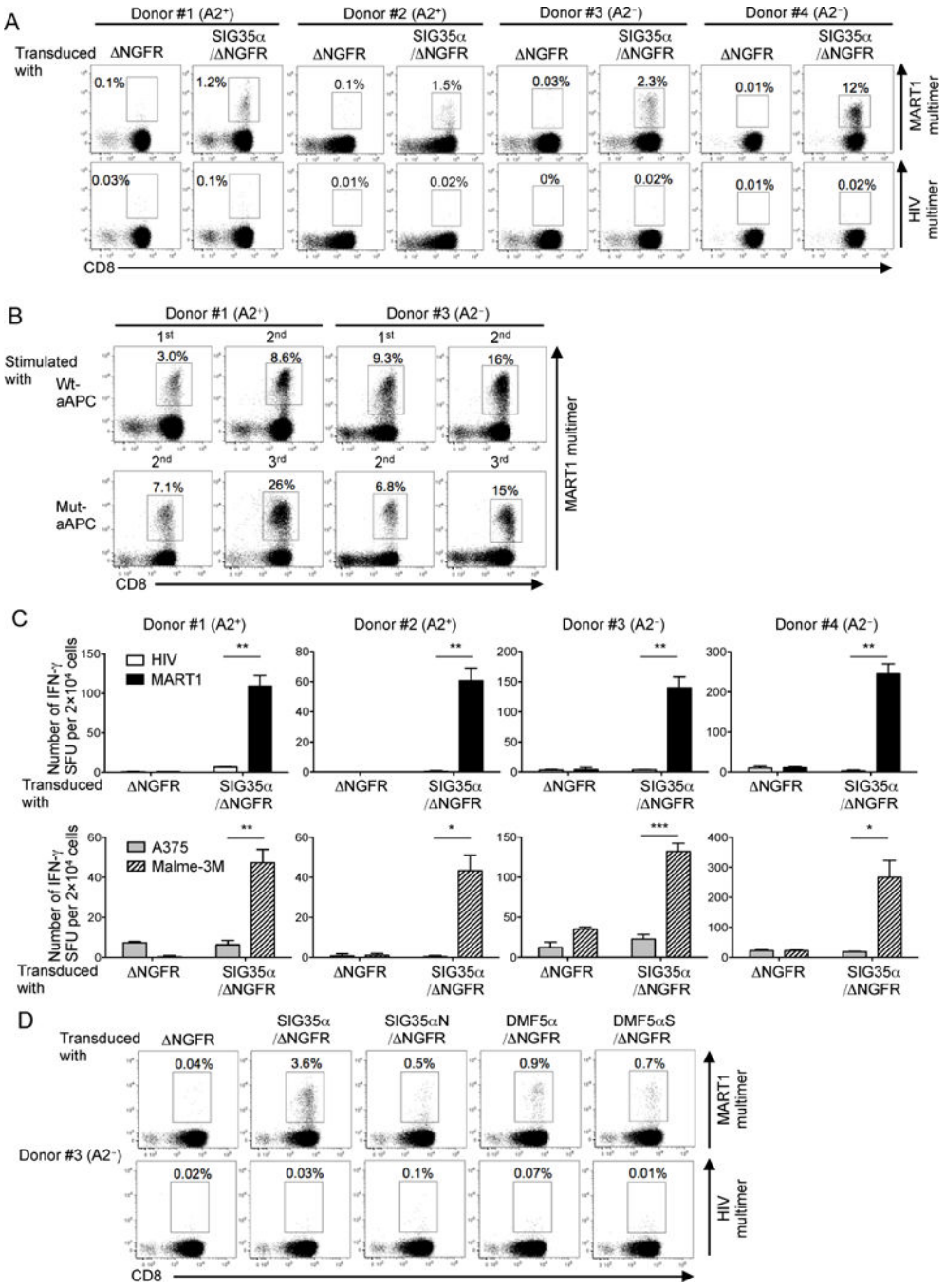

Fig. 2. Both HLA-A2$^+$ and A2$^-$ peripheral T cells can recognize A2/MART1 when transduced with chain-centric SIG35α

(*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 (#1 and #2) and two A2$^-$ 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-aAPC or mut-aAPC 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 days. Data depict A2/MART1 multimer staining performed following the first and second stimulations using wt-aAPC and the second and third stimulations utilizing mut-aAPC. Data shown are gated on $\Delta NGFR^+$ cells. Representative multimer-staining data from one of two HLA-$A2^+$ donors and one of two $A2^-$ donors are shown. (*C*), Peripheral T cells transduced with SIG35α are highly avid for A2/MART1 recognition. $CD8^+$ T cells following stimulation with wt-aAPC pulsed with wild-type A2/MART1 peptide were used as responder cells in IFN-γ ELISPOT analysis. T2 cells pulsed with 10 μg/ml A2/HIV control peptide or wild-type A2/MART1 peptide were used as stimulator cells (top). The $A2^+$ $MART1^-$ melanoma line, A375, and the $A2^+$ $MART1^+$ melanoma line, Malme-3M, were used as stimulator cells (bottom). Data shown are representative of two independent experiments. All experiments were carried out in triplicate and error bars depict SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (*D*), Peripheral $CD8^+$ T cells isolated from an $A2^-$ donor #3 were transduced with ΔNGFR, SIG35α/ΔNGFR, SIG35αN/ΔNGFR, DMF5α/ΔNGFR, or DMF5αS/ΔNGFR and stained by A2/MART1 multimer or A2/HIV multimer in conjunction with anti-CD8 mAb and anti-NGFR mAb as described in Fig. 1. Data shown are gated on $\Delta NGFR^+$ cells.

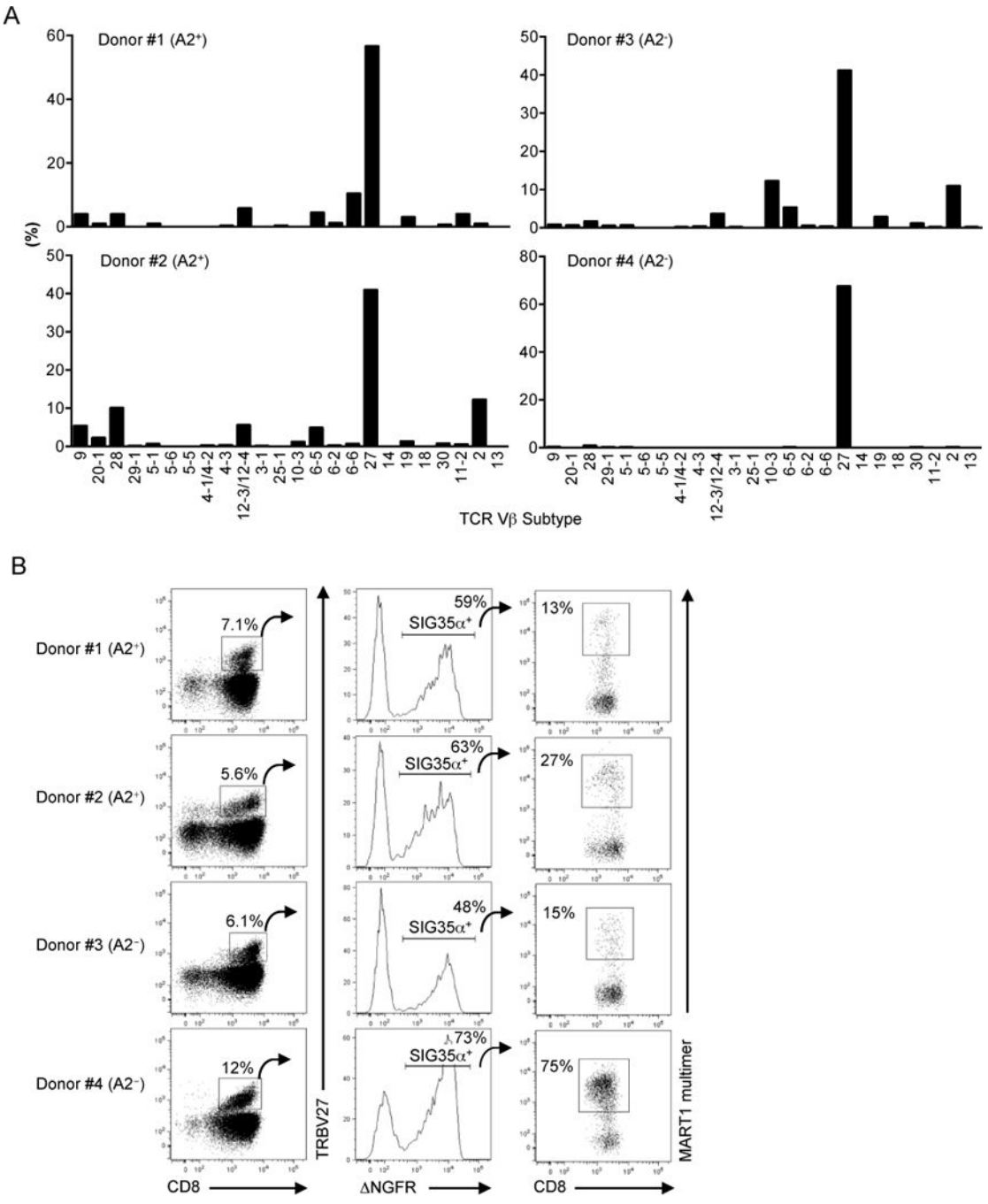

Fig. 3. SIG35α predominantly pairs with TRBV27 TCRβ chains to recognize A2/MART1

(*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 $multimer^+$ $CD8^+$ T cells expressing each subtype is shown. Data shown are gated on $\Delta NGFR^+$ 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.

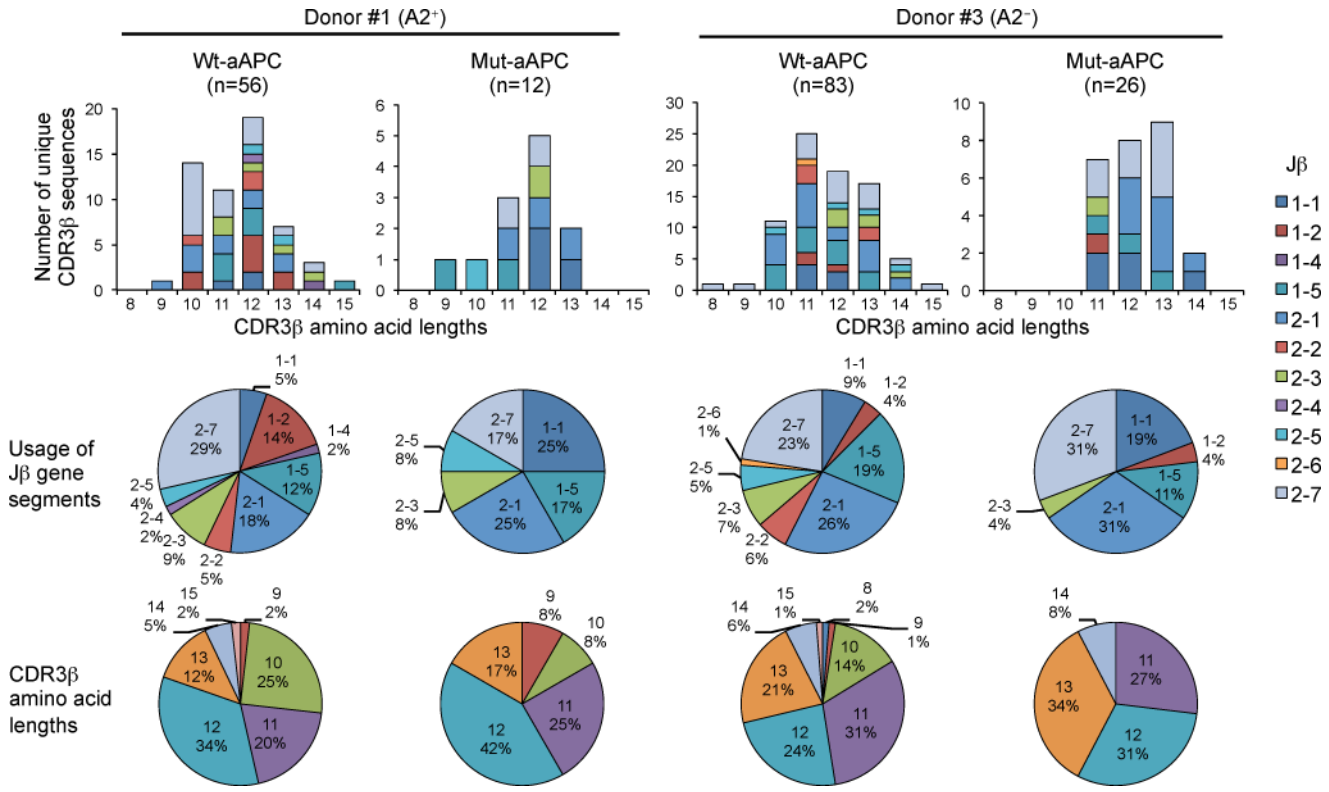

Fig. 4. TRBV27 TCRβ chains which recognize A2/MART1 when paired with SIG35α are highly heterogeneous and unique

SIG35α/ΔNGFR-transduced CD8$^+$ T cells from the HLA-A2$^+$ donor #1 and the A2$^-$ donor #3 were stimulated with wt-aAPC or mut-aAPC pulsed with wild-type A2/MART1 peptide. A2/MART1 multimer$^+$ CD8$^+$ T cells were collected by fluorescence activated cell sorting (>99% purity) and their TRBV27 CDR3β regions were amplified by PCR and sequenced after cloning. The number of unique CDR3β sequences (top), the relative usage of Jβ gene segments (middle), and the CDR3β amino acid lengths (bottom) are depicted separately for the A2$^+$ donor #1 (left) and A2$^-$ donor #3 (right). Data were analyzed by the aAPC used for stimulations, wt-aAPC vs. mut-aAPC, in each donor.

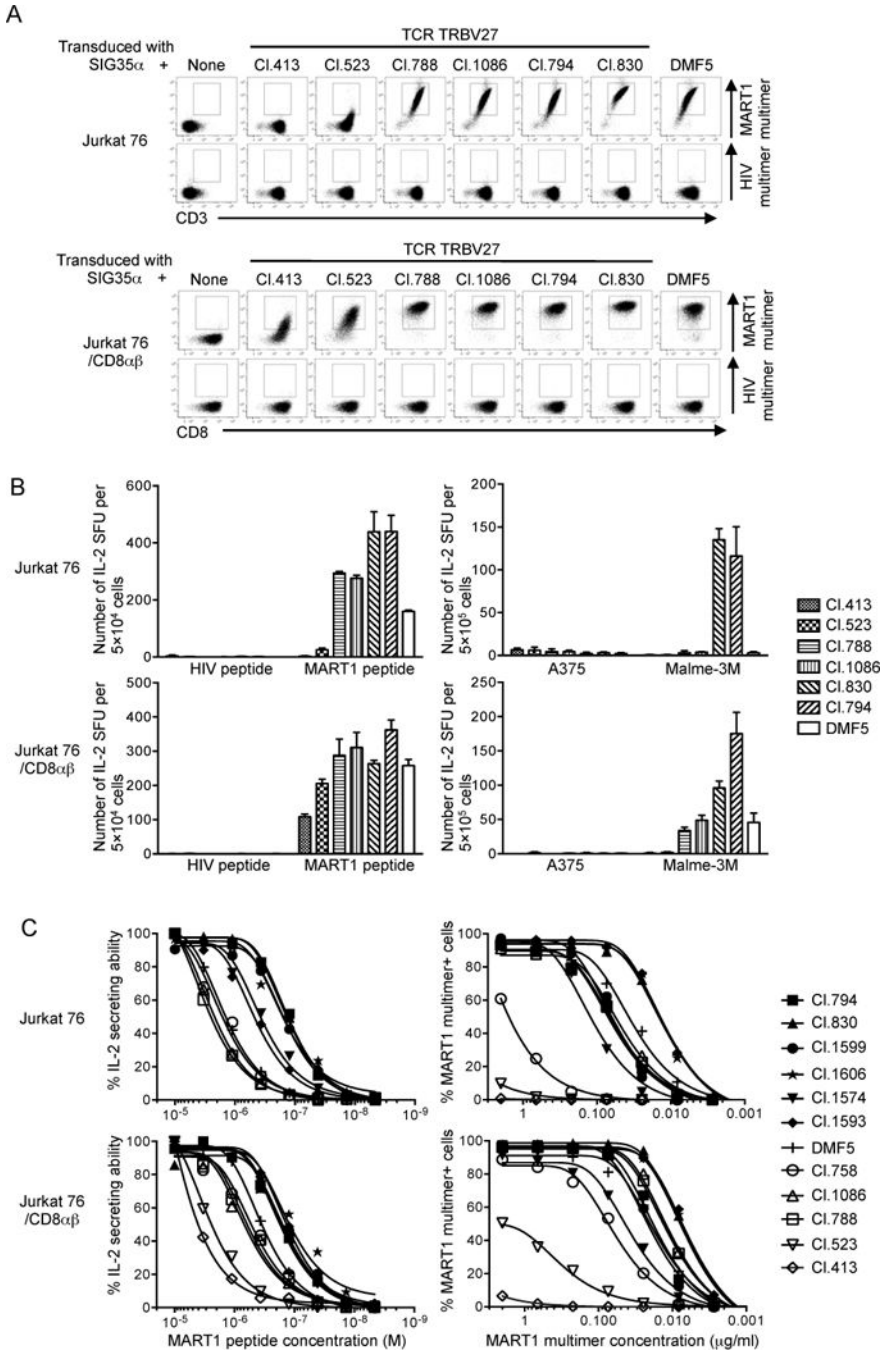

Fig. 5. The structural and functional avidity range of A2/MART1 TCRs consisting of SIG35α is very broad and further enhanced by the presence of CD8

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 eleven distinct TRBV27 TCRβ chains along with SIG35α or with DMF5αβ chains. (*A*), A2/MART1 TCRs reconstituted on Jurkat 76 or Jurkat 76/CD8αβ cells 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 5 transfectants are shown in Supplementary 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). The $A2^+$ $MART1^-$ melanoma line, A375, and the $A2^+$ $MART1^+$ melanoma line, Malme-3M were used as stimulator cells (right). All experiments were conducted in triplicate and error bars show SD. Data shown are representative of two independent experiments. (*C*), Reconstituted A2/MART1 TCRs possess a broad range of functional and structural avidities. Functional avidities of Jurkat 76 or Jurkat 76/CD8αβ cells expressing 11 different A2/MART1 TCRβ chains paired with SIG35α and DMF5 are depicted as % IL-2 secreting abilities determined by IL-2 ELISPOT assays using T2 cells pulsed with graded concentrations of wild-type A2/MART1 peptide as stimulator cells (left). Structural avidities of the same transfectants are shown as multimer staining percentages determined by staining with graded concentrations of A2/MART1 multimer (right). Data shown are representative of two independent experiments.

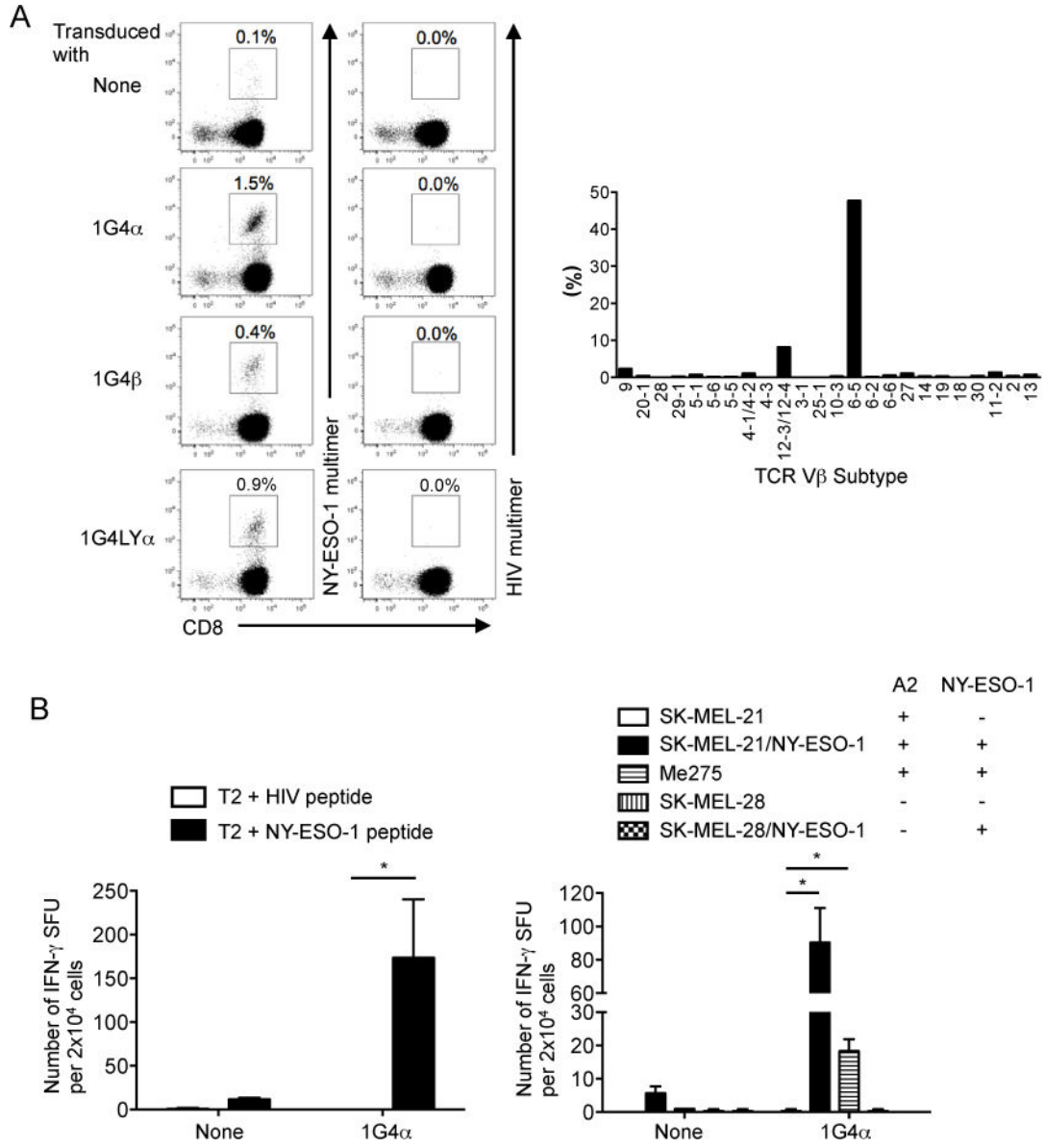

Fig. 6. TCR chain centricity is observed with other HLA-restricted antitumor TCRs

(*A*), Peripheral CD8$^+$ T cells transduced with 1G4α, 1G4β, or 1G4LYα recognize A2/NY-ESO-1. Peripheral CD8$^+$ T cells transduced with 1G4α, 1G4β, or 1G4LYα were stimulated with IL-21-secreting wt-aAPC pulsed with heteroclitic A2/NY-ESO-1 peptide once a week. Between stimulations, IL-2 (10 IU/ml) and IL-15 (10 ng/ml) were added every 3 days. Data for A2/NY-ESO-1 multimer staining conducted after second stimulation are shown (left). Data are representative of two donors. 1G4α-transduced CD8$^+$ T cells were costained with A2/NY-ESO-1 multimer, mAbs for TCR TRBV subtypes, and anti-CD8 mAb. The percentage of A2/NY-ESO-1 multimer$^+$ CD8$^+$ T cells expressing each subtype after second stimulation is shown (right). (*B*), Peripheral T cells transduced with 1G4α hemichain are highly avid for A2/NY-ESO-1 recognition. 1G4α-transduced or non-transduced CD8$^+$ T cells were stimulated with mut-aAPC pulsed with heteroclitic NY-ESO-1 peptide and used as responder cells in IFN-γ ELISPOT analysis. T2 cells pulsed with 10 μg/ml A2/HIV

control or A2/NY-ESO-1 peptide were used as stimulator cells (left). Various target cells, which did or did not express HLA-A2 and/or NY-ESO-1, were used as stimulator cells (right). Experiments were carried out in triplicate and error bars depict SD. *$P < 0.05$.

Table I

CDR3 sequences of TCRβ chains paired with SIG35α in A2/MART1 T cells

TRBV	CDR3β	TRBJ	References
27	CASSLLGGSTDTQYF	2-3	(24)
27	CASSPIDGLNTEAFF	1-1	(24)
27	CASSPSQGGNTEAFF	2-1	(24)
27	CASSDSTASSEQFF	2-1	(24)
27	CASSFNDEQFF	2-1	(23, 24)
5-1	CASSLSGSGDEQFF	2-1	(23, 24)

Table II

Sequencing results of TCR TRBV27 chains isolated from A2/MART1 multimer^{+} CD8^{+} T cells

	Wt-aAPC stimulation		Mut-aAPC stimulation		Number of shared clonotypes
Donor	**Number of unique clonotypes**	**Number of isolates sequenced**	**Number of unique clonotypes**	**Number of isolates sequenced**	
#1 (A2^{+})	56	190	12	19	0
#3 (A2^{-})	83	122	26	89	3
Total	139	312	38	108	3

Table III

Specific recognition of HLA-A2$^+$ MART1$^+$ tumor cells by Jurkat 76/CD8αβ reconstituted with A2/MART1 TCRs

			TRBV27 chain											
Stimulator cells	**HLA-A2**	**MART1**	**Cl.413**	**Cl.523**	**Cl.788**	**Cl.1086**	**Cl.758**	**Cl.1593**	**Cl.1574**	**Cl.1599**	**Cl.1606**	**Cl.830**	**Cl.794**	**DMF5**
A375	+	−	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A375/MART1	+	+	<10	<10	21 (6)	20 (5)	45 (7)	98 (8)	55 (9)	170 (3)	94 (10)	153 (8)	197 (10)	33 (3)
Malme-3M/siControl	+	+	<10	<10	18 (5)	19 (8)	64 (6)	73 (15)	60 (11)	143 (18)	76 (10)	99 (8)	151 (15)	39 (5)
Malme-3M/siMART1	+	Low	<10	<10	14 (5)	14 (6)	50 (6)	54 (5)	45 (9)	99 (10)	48 (4)	75 (9)	113 (11)	27 (5)
SK-MEL-28	−	+	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
SK-MEL-28/A2	+	+	<10	<10	34 (6)	31 (4)	67 (9)	101 (14)	81 (4)	128 (14)	70 (6)	115 (10)	147 (10)	40 (6)
SK-MEL-37	+	−	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
K562	−	−	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

Tumor cell recognition was assessed using various target cells, which did or did not express HLA-A2 and/or MART1, as stimulator cells in IL-2 ELISPOT assays. A half million Jurkat 76/CD8αβ cells, which were individually transduced with eleven distinct clonotypic TRBV27 TCRβ chains along with SIG35α or with DMF5αβ chains, were used as responder cells. Mean values of SFUs in triplicate samples are shown. SDs of triplicates are shown in parentheses.

Table IV

Recognition of MART1–related peptides by Jurkat 76/CD8αβ reconstituted with A2/MART1 TCRs

			TRBV27 chain											
No.	**Protein**	**Peptide sequence**	**Cl.413**	**Cl.523**	**Cl.788**	**Cl.1086**	**Cl.758**	**Cl.1593**	**Cl.1574**	**Cl.1606**	**Cl.1599**	**Cl.830**	**Cl.794**	**DMF5**
1	KIAA0935	RVTDEAGHPV	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
2	cMOAT2	NVADIGLHDV	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
3	SLC1A1	VLTGLAIHSI	<10	<10	<10	<10	<10	<10	29	42	<10	<10	<10	<10
4	P47	RISDIRLFIV	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
5	Prostaglandin transporter	LLAGIGTVPI	100	248	153	100	205	272	296	277	241	189	283	174
6	ABC transporter MOAT-C	RISDIGLADL	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
7	KIAA0735	LISGIGIGGA	<10	33	<10	<10	<10	32	100	111	31	23	36	<10
8	Hypothetical 20 kD protein	RISAIILHPN	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
9	Endothelin-1 receptor	RVQGIGIPLV	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
10	G-protein coupled receptor RE2	RITDLGLSPH	<10	64	35	<10	124	43	215	213	168	17	248	80
11	IGHG1	RLSELAIFGV	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
12	Monocarboxylate transporter 8	AVAFIGLHTS	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
13	MRP3	NVADIGFHDV	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
14	Wild type MART1	AAGIGILTV	20	102	100	163	198	270	235	216	218	112	272	144

Peptide recognition was assessed using T2 cells loaded with 10 μg/ml of the indicated peptide as stimulator cells in IL-2 ELISPOT assays. Thirteen MART1-related peptides were reported by Dutoit et al. (53). Fifty thousand Jurkat 76/CD8αβ cells, which were individually transduced with eleven distinct TRBV27 TCRβ chains along with SIG35α or with DMF5αβ chains, were used as responder cells. Mean values of SFUs in triplicate samples are shown.

Table V

Functional and structural avidities of the A2/MART1 TCRs

Clone	Donor	aAPC used for stimulation	TRBV	CDR3β	TRBJ	Functional avidity* without CD8	Functional avidity with CD8	Structural avidity† without CD8	Structural avidity with CD8
						EC50 (μM)	EC50 (μM)	EC50 (μg/ml)	EC50 (μg/ml)
Cl.794	#3 ($A2^-$)	Mut-aAPC	27	CASSLLGDYGYTF	1-2	0.12	0.16	0.06	0.02
Cl.830	#3 ($A2^-$)	Mut-aAPC	27	CASSLGGAYEQYF	2-7	0.13	0.14	0.01	0.006
Cl.1599	#1 ($A2^+$)	Mut-aAPC	27	CASSFLGAMAEAFF	1-1	0.14	0.16	0.06	0.02
Cl.1606	#1 ($A2^+$)	Mut-aAPC	27	CASSLLGSYEQYF	2-7	0.16	0.12	0.01	0.006
Cl.1574	#1 ($A2^+$)	Mut-aAPC	27	CASSPWERINTEAFF	1-1	0.35	0.15	0.1	0.03
Cl.1593	#1 ($A2^+$)	Mut-aAPC	27	CASGNNQPQHF	1-5	0.44	0.14	0.01	0.006
DMF5‡			6-4	CASSLSFGTEAFF	1-1	1.4	0.33	0.03	0.02
Cl.758	#3 ($A2^-$)	Wt-aAPC	27	CASSPRLAGDGELFF	2-2	1.6	0.46	2.7	0.06
Cl.1086	#3 ($A2^-$)	Wt-aAPC	27	CASSLHGPGGYTF	1-2	2.4	0.63	0.04	0.01
Cl.788	#3 ($A2^-$)	Wt-aAPC	27	CASGPSYEQYF	2-7	2.9	0.57	0.05	0.01
Cl.523	#3 ($A2^-$)	Wt-aAPC	27	CASGSYEQYF	2-7	n.m	2.7	n.m	0.3
Cl.413	#1 ($A2^+$)	Wt-aAPC	27	CASSVFGGDMGEKLFF	1-4	n.m	10	n.m	n.m

n.m, not measurable.

*Functional avidity, expressed as EC50 in μM, was defined as the concentration of peptide required to achieve 50% of maximal response.

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

‡DMF5 is a high avidity A2/MART1 TCR described in ref. (48, 57)