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T-cell receptors engineered de novo for peptide specificity can mediate optimal T-cell activity without self cross-reactivity

Preeti Sharma^{1,3}, Daniel T. Harris¹, Jennifer D. Stone^{1,2}, David M. Kranz^{1,3}

¹Department of Biochemistry, University of Illinois, Urbana, IL 61801, USA

²Present Address: AbbVie, North Chicago, IL 60064, USA

Abstract

Despite progress in adoptive T-cell therapies, identification of targets remains a challenge. Although chimeric antigen receptors recognize cell-surface antigens, T-cell receptors (TCRs) have the advantage that they can target the array of intracellular proteins by binding to peptides associated with MHC products (pepMHC). Although hundreds of cancer-associated peptides have been reported, it remains difficult to identify effective TCRs against each pepMHC complex. Conventional approaches require isolation of antigen-specific CD8⁺ T cells, followed by TCR $\alpha\beta$ gene isolation and validation. To bypass this process, we used directed evolution to engineer TCRs with desired peptide specificity. Here, we compared the activity and cross-reactivity of two affinity-matured TCRs (T1 and RD1) with distinct origins. T1-TCR was isolated from a melanoma-reactive T-cell line specific for MART-1/HLA-A2 whereas RD1-TCR was derived *de novo* against MART-1/HLA-A2 by *in vitro* engineering. Despite their distinct origins, both TCRs exhibited similar peptide fine-specificities, focused on the center of the MART-1 peptide. In CD4⁺ T cells, both TCRs mediated activity against MART-1 presented by HLA-A2. However, in CD8⁺ T cells, T1 but not RD1 demonstrated cross-reactivity with endogenous peptide/HLA-A2 complexes. Based on the fine specificity of these and other MART-1 binding TCRs, we conducted bioinformatics scans to identify structurally similar self-peptides in the human proteome. We showed that the T1-TCR cross-reacted with many of these self-peptides whereas the RD1-TCR was rarely cross-reactive. Thus, TCRs like RD1, generated *de novo* against cancer antigens, can serve as an alternative to TCRs generated from T-cell clones.

Keywords

Adoptive T-cell therapy; T-cell receptor; peptide specificity; affinity thresholds; TCR engineering

Introduction

T-cell receptors ($\alpha\beta$, TCRs) expressed on T cells recognize diverse self-, foreign- and mutated peptides presented by proteins encoded by genes of the major histocompatibility

³**Corresponding Authors:** Dr. Preeti Sharma and Dr. David M. Kranz, Department of Biochemistry, University of Illinois, 600 S Mathews Ave, Urbana, IL 61801, Phone: 217-244-2823; 217-244-2821, Fax: (217) 244-5858, sharma39@illinois.edu; d-kranz@illinois.edu.

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complex (MHC) (1). Class I MHC molecules typically present peptides that are 8 to 10 amino acids long. Thus, the potential diversity of such peptide/HLA-A2 (pep/HLA-A2) complexes alone can be as high as 10^{13} , exceeding the number of TCRs in the human body (2). A single TCR is capable of cross-reacting with multiple peptides in order to enable T-cell immunity (3). In order to avoid autoimmune reactivity that might be promoted by such cross-reactivity, T cells undergo a process of central tolerance in which T cells that bind with too high of an affinity for self-peptide/MHC are deleted in the thymus (4). The process yields a repertoire of TCRs on peripheral CD8⁺ T cells poised to mediate activity when bound with relatively low affinity (K_D values in the sub-micromolar range), in a CD8-dependent mechanism, to a 'foreign' peptide/class I MHC antigen.

The term 'foreign', as used here, includes mutated self-peptides (such as neoantigens in cancer) or non-self antigens such as those expressed by infectious agents. Aberrantly upregulated self-peptides, in which the density of the self-peptide/MHC complex is greater than normal, can also be recognized by some T cells which were tolerized only to the lower amounts of the pepMHC. This in fact represents the scenario of many studies of cancer-associated antigens (5). In general, TCR binding affinity for 'foreign' antigens is several fold higher than TCR binding affinity for self-peptide antigens (6,7), likely due to the process of central tolerance. These lower affinity TCRs typically require more pepMHC on target cells to induce a CD8⁺ T-cell response; due to their CD8-dependence they are also unable to mediate activity of CD4⁺ T cells (8).

To increase the sensitivity of TCRs against cancer-associated self-peptides, and to enable class I MHC-restricted TCRs to mediate activity independent of CD8 (i.e. in CD4⁺ T cells), it is possible to increase TCR affinity by various methods. These methods include *in vitro* engineering using yeast display (9), phage display (10), T-cell display (11,12), structure-guided mutagenesis (13,14), the isolation of TCRs from CD8-deficient or alloreactive T cells (15,16), or the identification of TCRs from *in vivo* selections (17). These approaches can yield TCRs that endow T cells with optimal sensitivity and functionality in CD4⁺ T cells. The challenge with affinity matured TCRs is that the increased affinity can also yield functional cross-reactivity with structurally similar peptide antigens (18), especially with the synergism provided by CD8 (19). If these antigens are present on healthy tissue, TCR-mediated cross-reactivity can present safety issues. Examples of this risk have been described in patients receiving T cells with affinity-enhanced TCRs. One TCR was against HLA-A01-restricted MAGE-A3 (EVDPIGHLY); treatment with T cells expressing this TCR led to two deaths, likely due to cross-reactivity with a structurally-similar self-peptide (ESDPIVAQY) derived from the protein titin that was expressed in cardiac tissue (20). Another TCR was against a MAGE-A3 epitope restricted by HLA-A2, which cross-reacted with the MAGE-A12 epitope in the nervous system, leading to another two deaths (21).

These incidents prompted addition of a standard safety screen of the proteome for structurally similar self-peptides that might predict potential problems. Additionally, TCRs expressed on the T-cell surface have an "optimal affinity window" beyond which affinity-enhancement does not translate to increased potency but instead reduces specificity (19,22). Thus, efforts are directed toward designing TCRs (or tuning their affinity) at the low end of this optimal window for use in adoptive T-cell therapy. TCR affinity thresholds against class

I antigens differ for CD4⁺ and CD8⁺ T cells due to the participation of CD8, yet driving both CD4⁺ and CD8⁺ T cells against the tumor antigens can be useful (e.g. (23,24)). Hence, TCR affinity for cognate pepMHC needs to be tuned for optimal responses in both CD4⁺ and CD8⁺ T cells, without cross-reactivity to self-antigens (7). In contrast, TCRs in a soluble therapeutic format can benefit from having higher affinity and exhibit dose-dependent responses, as with other soluble therapies including antibodies (25).

Despite these challenges, TCRs remain an attractive approach for adoptive T-cell therapy due to their ability to target potentially any antigen derived from intracellular proteins (26). The current process for identifying, validating, and optimizing a therapeutic TCR remains a bottleneck for the development of TCR-mediated therapies. The conventional approach involves production of antigen-specific T cells and multiple additional steps: 1) Stimulation of PBMCs with peptide antigen or isolation of tumor-infiltrating lymphocytes (TILs) from tumors, 2) *In vitro* expansion of T cells and assessment of peptide specificity, 3) Isolation of TCR α and β genes from T-cell clones or single cell PCR, 4) Transfer of the candidate TCR genes into T cells for verification of specificity with the peptide antigen, 5) TCR affinity engineering by *in vitro* techniques, or screens of many TCRs, for optimal affinity/activity (e.g. (27–30)).

The effort and time needed to obtain peptide-specific TCRs against each of the hundreds to thousands of cancer-associated peptide/HLA complexes (5,26) and patient-specific neoantigens (27) prompted our lab to consider an alternative, higher throughput discovery strategy. In this approach, TCRs are generated *de novo* using the principles and speed of directed evolution (31). Previously, we used directed evolution and a yeast display system to switch the specificity of a TCR from a viral peptide (Tax) to the melanoma antigen MART-1 (31). For this approach to be applied to adoptive T-cell therapies, the TCRs should have specificities similar to conventional TCRs and should mediate functional activity in CD4⁺ and CD8⁺ T cells, without dangerous peptide cross-reactivity. Here we show that this is the case with the TCR called RD1 that was engineered *de novo* against MART-1/HLA-A2. The RD1 TCR exhibited desirable properties even compared to a TCR called T1 that was generated against MART-1/HLA-A2 by conventional means (Fig. 1). Hence, directed evolution can be used to identify therapeutic TCRs with ‘designer’ peptide specificities without the need for isolation of T-cell clones against each antigen.

Materials and Methods

Yeast Display, reagents and flow cytometry

T1 and RD1 TCRs were expressed as single-chain TCRs in yeast display vector, pCT302 (31,32). Soluble, monomeric HLA-A2 was prepared *in vitro* by refolding HLA-2 heavy chain and β 2-microglobulin in the presence of UV-cleavable peptide, KILGFVFIJ (J is 3-amino-3-(2-nitro)phenyl-propionic acid) (33). The HLA-A2 heavy chain sequence included a biotinylation substrate sequence for *in vitro* biotinylation by BirA enzyme (Avidity, LLC). Peptides (anchor-modified, MART-1: ELAIGILTIV and single amino acid variants; WT1: RMFPNAPYL, UV-cleavable peptide: KILGFVFIJ and various self-peptides (Supplementary Tables S1 and S2) were synthesized by GenScript Inc. For binding experiments, pep/HLA-2 monomers were prepared by UV-mediated peptide exchange (33).

Tetrameric pep/HLA-A2 were prepared by mixing biotinylated monomers with streptavidin-phycoerythrin (streptavidin-PE) (catalog number 554061 from BD Biosciences) in a 4:1 molar ratio.

Binding of yeast-displayed single chain TCRs to pep/HLA-A2 was assessed by incubating cells with various concentrations (4 nM to 1 μ M) of monomeric pep/HLA-A2 for 1 hour. After washing, bound pep/HLA-A2 complexes were detected by staining with streptavidin-PE (diluted 1:100 from 0.5 mg/ml stock) by flow cytometry. For tetramer binding, cells were stained with 1 μ M fluorescent pep/HLA-A2 tetramers for an hour, washed and then analyzed by flow cytometry. Fluorescence profile of cells stained with streptavidin-PE only (negative control) was used to set the gates.

T-cell isolation and transduction with T-cell receptor

T1 and RD1 full-length TCR genes were cloned in-frame with murine constant region genes in pMP71 retroviral vector (34–36). Plat-E retroviral packaging cells were plated at 10^6 cells per well in complete DMEM media (with blasticidin and puromycin) on a poly-L-lysine coated, six-well plate, and allowed to grow at 37°C, 5% CO₂. After 24 hours, cells were incubated with 10 μ g plasmid DNA or mock (no DNA) in the presence of Lipofectamine™ 2000 transfection reagent (Invitrogen™). After 4 hours, cells were washed and cultured in complete IMDM media. After 48 hours, retroviral supernatants were harvested and filtered through 0.45 μ m filter.

CD4⁺ or CD8⁺ T cells were isolated from the spleens of C57BL/6 mice using the Dynabeads® Untouched™ mouse T cells isolation kit (Invitrogen™). Cells were activated for 24 hours with anti-CD3/anti-CD28 Dynabeads (Invitrogen™) in the presence of recombinant, murine IL-2 (Roche). Activated CD4⁺ or CD8⁺ T cells were transduced using retroviral supernatant containing T1-TCR or RD1-TCR or mock (no DNA) in the presence of Lipofectamine 2000 and IL-2, on RetroNectin- (Takara Bio USA, Inc.) coated, 24-well, non-tissue culture treated plates, and cultured for 72 hours. After 48 hours, transduced T cells were split 1:1 and transduction efficiency was assessed by staining cells with MART-1/HLA-A2 tetramers.

T-cell activation assays

For activation assays, splenocytes isolated from HLA-A2/D^b transgenic C57BL/6 mice (AAD⁺ mice, expressing α 1 and α 2 domain of HLA-A2, fused with α 3 domain of H-2D^b) (37), or human cell lines (T2, HEK293F, MCF7, Jurkat) were used as antigen presenting cells (APCs). All cell lines were thawed from liquid nitrogen storage and cultured for approximately a week before using in the experiments. HEK293F, MCF7 and Jurkat were a gift from Dr. Erik Procko (University of Illinois), Dr. Erik Nelson (University of Illinois) and Dr. Hans Schreiber (University of Chicago) respectively. The cell lines were not authenticated in the past year, or tested for Mycoplasma. T2 cells are TAP deficient, and hence do not present physiological amounts of some endogenous pepMHC complexes on their surface. Expression of HLA-A2 on APCs was assessed by staining cells with FITC conjugated anti-human HLA-2 antibody (clone BB7.2; catalog number 551285 from BD Biosciences) and analysis by flow cytometry. T1-TCR, RD1-TCR, or mock- transduced T

cells (CD4⁺ or CD8⁺; 55,000 to 75,000) were incubated with equal numbers of APCs in the absence or presence of various concentrations of peptides for 24 hours. In some assays, T cells and APCs were mixed at various effector:target (E:T) cell ratios, in the absence of peptide. After 24 hours, supernatants were harvested and analyzed for the presence of interferon- γ (IFN γ) using an ELISA (Invitrogen™) following manufacturer's instructions.

Results

Comparison of two TCRs generated by distinct approaches

Here we compared two TCRs, T1-S18.45 and RD1-MART1^{HIGH}, that bind to the same cancer antigen, MART-1/HLA-A2 (Fig. 1). Here onwards, these TCRs are referred to as T1 and RD1 TCRs, respectively. RD1 TCR was derived previously from the TCR known as A6 completely by de novo engineering, using libraries of CDR mutations to switch peptide specificity. Thus, the A6 TCR was converted from binding to the peptide Tax (LLFGYPVYV) to the TCR RD1 that bound to MART-1 (ELAGIGILTV)/HLA-A2 complexes (31). The structure of the RD1-TCR:MART-1/HLA-A2 complex (Fig. 2A) showed that it exhibited the canonical diagonal orientation (35). T1 TCR was derived by conventional means from a human T-cell clone (32). Both T1 and RD1 TCRs were also each affinity matured to bind MART-1/HLA-A2 with sub-micromolar affinities using standard CDR mutagenesis followed by yeast display and selection (K_D of 100 ± 5 nM, and 500 ± 5 nM, for T1 and RD1 respectively) (31,32,35).

Although T1 and RD1 TCRs recognize the same pep/HLA-A2 complex, they differ in amino acid sequences at four of their six CDRs (Fig. 2B), with different CDR3 α and CDR3 β loops. Notwithstanding the sequence differences in CDR3s, these loops were required for MART-1/HLA-A2 binding, as shown by deep mutational scans of every CDR of RD1 (35) and T1 (38), in addition to the structure of RD1 TCR (Fig. 2A) (35). Side-by-side analysis of the average reduction in prevalence for substitutions among residues in each CDR are shown in Fig. 2C. Although both TCRs used residues within multiple CDRs to contribute binding energy, the two CDR3 regions contributed the most binding energy. Residues in framework (FR) regions were used as internal controls for impact on protein folding and stability.

TCR RD1 exhibits same peptide-binding signature as conventional TCR T1

MHC-bound peptides require several side chains for binding to MHC, allowing the remainder to be exposed at the surface of the complex for interactions with the TCR. These exposed residues are expected to confer specificity for TCR binding, which impacts the cross-reactivity of the TCR with other peptides. These interactions affect the development of therapeutic TCRs as cross-reactivity leads to toxicity associated with recognition of structurally related self-peptides during adoptive T-cell therapy (20). Since CDR3 loops of TCRs dock predominantly over the peptide in TCR:pepMHC structures (Fig. 2A, **inset**) (39), we were interested in determining if the peptide side chains of MART-1 contributed different binding energies to the interactions with T1 and RD1 (i.e. they exhibited different fine-specificities). To examine this, we used a panel of MART-1 variant peptides (conserved amino acid or alanine substitutions) to study each residue in MART-1. For quantitative

binding analysis, we used T1 or RD1 TCRs displayed on the surface of yeast and performed titrations with monomeric peptide/HLA-A2 complexes (Fig. 3, Supplementary Fig. S1).

Our results demonstrated that the specificity of MART-1/HLA-A2 binding to both TCRs was similar (Fig. 3A, B), despite their distinct origins. Binding energy was primarily governed by amino acids that were located at the center of the peptide (i.e., G4, I5, G6, I7 and L8), with maximum impact evident by substitution of residues that pointed toward the TCR in structures of MART-1 with RD1-TCR or other TCRs specific for MART-1/HLA-A2 (MeI5 or DMF5) (Fig. 3C) (35,40,41). Changing residues at the N-terminus (E1, L2, A3) as well as C-terminus (T9, V10) to either conserved amino acids or alanine did not significantly impact binding affinity of either TCR (Fig. 3A, B), although substitutions of these residues could of course impact antigen presentation by HLA-A2.

Given the role of glycine in backbone flexibility, binding by the TCRs to glycine mutants (G4S/A or G6S/A) was particularly interesting. Serine substitutions abrogated binding whereas alanine substitutions allowed some binding by either one or both TCRs (Fig. 3A, B). Reduction in binding to I5L or I5A mutants by both TCRs indicated that these substitutions, and substitution of the two flanking glycines, disrupted the TCR-pepMHC interaction. Although conservative substitutions of I7 and L8 were tolerated, alanine substitutions showed some reduction in binding, indicating these also play a role in the specificity of the TCRs. As the isoleucine side chain at position 7 in MART-1 points toward the HLA-A2, substitution to an alanine may act indirectly by altering the exposed surface of the peptide or the HLA-A2 helices. Collectively, these results show that the two TCRs have similar specificity, despite their distinct origins and CDR3 sequences.

T1 and RD1 TCRs mediate MART-1-specific reactivity in CD4⁺ T cells.

The sub-micromolar affinities of the T1 and RD1 TCRs suggest that they may be capable of driving the activity of CD4⁺ T cells, without the need for CD8 (7). To examine this possibility, we isolated CD4⁺ T cells from C57BL/6 mice, activated the cells with anti-CD3/anti-CD28 beads, and transduced them with T1 or RD1 TCR. Staining with MART-1/HLA-A2 tetramers showed that about 50% of the cells were transduced in each case (Fig. 4A). To examine activity, the transduced CD4⁺ T cells were stimulated with various concentrations of peptides in the presence of splenocytes from HLA-A2/D^b transgenic mice. These antigen-presenting cells (APCs) would be capable of stimulating mouse T cells with the participation of mouse CD8 due to the fused $\alpha 3$ domain of mouse class I H-2D^b. T1-TCR- and RD1-TCR-transduced CD4⁺ T cells were both activated ($EC_{50} \sim 10$ nM) by MART-1 peptide (Fig. 4B,C). Hence, the affinity of each TCR was sufficient to mediate activity in the absence of CD8, and there was no evidence of basal activity generated by endogenous peptides presented by the HLA-A2⁺ APCs.

T1, but not RD1, on CD8⁺ T cells mediates cross-reactivity with endogenous peptides

TCRs with an affinity for a class I complex that is sufficient to fully activate CD4⁺ T cells also run the risk of mediating CD8⁺ T-cell activity against structurally similar endogenous peptides. This possibility is due to the binding and signaling synergy that is attributable to the CD8 molecule. To examine the activity of T1 and RD1 in this regard, we isolated CD8⁺

T cells from C57BL/6 mice, activated the cells with anti-CD3/anti-CD28 beads, and transduced them with T1 or RD1 TCR (Fig. 5A). T1-transduced CD8⁺ T cells were activated by AAD⁺ APCs even in the absence of MART-1 peptide (Fig. 5B). This finding is reminiscent of previous studies that have shown higher affinity TCRs mediate CD8-dependent activity by cross-reacting with endogenous peptide(s)/MHC (19,22). These results are also in accordance with lower affinity thresholds of class I-restricted TCRs, and their greater sensitivity, in CD8⁺ T cells compared to CD4⁺ T cells (8,42). In the case of the T1-transduced CD8⁺ T cells, this activity was maximal in that the addition of exogenous MART-1 peptide did not raise the amount of released IFN- γ above this AAD⁺ APC background. In contrast to the result with the T1 TCR, RD1-transduced CD8⁺ T cells did not exhibit stimulation by AAD⁺ APCs alone, but it did exhibit a dose-dependent response to MART-1 peptide (EC₅₀ ~ 1 nM) (Fig. 5C).

Assessment of T1- and RD1-transduced CD8⁺ T-cell reactivity with human cell lines

In order to determine if T1-transduced CD8⁺ T cells would exhibit similar recognition of endogenous peptides presented by human APCs, we measured activation of T1- and RD1-transduced CD8⁺ T cells in the presence of human cell lines T2, HEK293F, MCF7 and Jurkat. T2, HEK293F, and MCF7 were confirmed to express HLA-A2 by flow cytometry, with T2 expressing the most (Fig.6A). Jurkat was HLA-A2-negative by flow cytometry. As observed with AAD⁺ APCs, T1-transduced CD8⁺ T cells were also activated with the T2 APC (and to a lesser extent with HEK293F) indicating recognition of endogenous peptides presented by HLA-A2 on these human cell lines (Fig 6B, C). The extent of activation was correlated with the amount of HLA-A2, with MCF7 and Jurkat showing no stimulatory ability (Fig 6D, E). In contrast to T1, RD1-transduced CD8⁺ T cells were not stimulated by T2 or HEK293F cells (Fig 6B, C), consistent with the observation using AAD⁺ APCs.

Bioinformatic identification of structurally similar self-peptides

It is now standard practice to conduct a bioinformatic search for peptides that might be structurally similar to a cognate antigenic peptide, thus raising possible safety issues due to cross-reactivity in normal tissues. We initially used a “tight” search string to scan human proteome (UP000005640 downloaded from UniProtKB database) based on similar binding signatures of the two TCRs from our experimental data (Fig. 3A,B). In this case, the string contained residues at each position in MART-1 that allowed binding to either one or both TCRs ([AIL][AG][AGS][IL][AGS][AIL][AIL][AST][AIV] for the nonamer scan or [ADE][AIL][AG][AGS][IL][AGS][AIL][AIL][AST][AIV] for the decamer scan). These “tight” scans yielded 46 matches, with 19 unique sequences (excluding MART-1) in the human proteome. Because HLA-A2/D^b transgenic mice could serve as a pre-clinical model to assess potential cross-reactivities, we also scanned these 19 hits against mouse proteome (UP000000589, downloaded from UniProtKB database) and showed that 12 of them (63%) were identical in the mouse proteome. These peptides were screened for predicted binding to HLA-A2 by various binding algorithms (SYFPEITHI, BIMAS, ANN, SMM and NetMHC) to identify possible HLA-A2 presented peptides in mouse (Supplementary Table S1).

Two of the peptides had NetMHC-predicted binding affinities for HLA-A2 that were higher than that of MART-1 nonamer (K_D predicted = 2254 nM): a peptide from Transmembrane

protein 74B (TM74B-9: IAGLGLLTV) and a peptide from RNA-binding protein Nova-1 (ALGSLAAATA) with predicted K_D values of 2036 and 1772 nM, respectively. TM74B-9 was most similar to MART-1 nonamer (AAGIGILTV) as it was identical in 6 out of 9 positions and homologous in 2 of the other positions. We also subjected the sequence of the TM74B protein from the human proteome to immunoproteasome cleavage predictions using an online tool available on the Immune Epitope Database and Analysis Resource (IEDB) (<http://tools-int-01.liai.org/processing/>). Of the 683 octamer, nonamer, and decamer peptides identified in this cleavage screen, we synthesized three variants of the peptide sequence related to MART-1 (TM74B-8a, 8b or 9) which differed in length and in predicted binding to HLA-A2. We measured their binding, as peptide-exchanged HLA-A2 monomers or tetramers, to T1 and RD1 TCRs (Supplementary Fig. S2). In addition, we also examined binding of the TM74B peptide variants to higher affinity mutants of the T1 and RD1 TCRs (T1-TCR^{HIGH}, RD1-TCR^{HIGH}) that were engineered previously (38,43) (Supplementary Fig. S2). This allowed us to compare the binding of HLA-A2 complexes of the TM74B peptide variants and MART-1 to a panel of TCRs that ranged in affinity for MART-1/HLA-A2 from 2 nM to 500 nM. Binding of T1 to 1 μ M TM74B-9/HLA-A2 monomer was detected as a slight peak shift, (Supplementary Fig. S2A), but binding of T1 and RD1 to TM74B-9/HLA-A2 tetramers was higher (Supplementary Fig. S2B). This result indicates that both TCRs bound to the TM74B nonamer but with low affinity. Although both T1-TCR^{HIGH} and RD1-TCR^{HIGH} exhibited similar dissociation constants for MART-1/HLA-A2 (K_D = 2 nM, (38,43)), binding to TM74B-8b/HLA-A2 and TM74B-9/HLA-A2 as monomers was only detected with T1-TCR^{HIGH} but not RD1-TCR^{HIGH} (Supplementary Fig. S2A). In contrast, binding to TM74B-9/HLA-A2 tetramers was detected with both TCRs (Supplementary Fig. S2B). These results suggest that both the T1 and RD1 TCRs (and their high-affinity variants) have convergent cross-reactivity on the same structurally related self-peptide TM74B, but the RD1 TCR has a lower affinity than the T1 TCR.

To further expand our assessment of possible cross-reactivity, we conducted an additional scan using a string that was designed based on the central “GIG” motif of MART-1 peptide, key to TCR binding (Fig. 3 A,B; Supplementary Fig. S1) and anchor residue requirements for peptide binding to HLA-A2 (NetMHC 4.0). In addition, prior information regarding binding signatures of two other MART-1 directed TCRs (Me15 and DMF5) (44,45) were also included in the string design, thus yielding [X][LMI][G][I][G][AFILMSVW][AGIKLMNVWY][ADIKLMNPQRSTV][LVIMA] and [DEFGHIKLMNS][LMI][AEFGKLMPSTW][G][I][G][AFILMSVW][AGIKLMNVWY][ADIKLMNPQRSTV][LVIMA] as 9-mer and 10-mer strings. Human proteome scans with these strings yielded 64 and 30 hits respectively, of which 25 nonamers and 11 decamers were unique (Supplementary Table S2). To examine if these additional human endogenous peptides would stimulate T1 or RD1 transduced CD8⁺ T cells, we measured their stimulation with peptide-pulsed HLA-A2⁺ T2 cells, reasoning that these APCs would provide the most sensitive strategy to see cross-reactivity above any basal endogenous activity (with T1). T1 transduced CD8⁺ T cells were stimulated by 20 of the 36 structurally similar self-peptides (1 μ M), whereas RD1 was only stimulated by one of the peptides (Fig. 7). Stimulation at 100-times higher concentrations verified these results (Supplementary Fig. S3). These results further indicated that the T1 TCR is far more cross-reactive than the RD1 TCR with

potential endogenous peptides, and that the de novo engineered RD1 TCR represents a preferred alternative to the T1 TCR, based on its antigen specificity and reduced cross-reactivity.

Discussion

More than 450 peptide antigens linked to human cancer have been identified (5,46). Although the search for those antigens that will be sufficiently cancer specific remains a challenge, there are also bottlenecks associated with identification of TCRs to target them by adoptive T-cell (47,48) or soluble therapies (25). To isolate and validate a TCR against a specific cancer antigen by T-cell cloning or selection followed by affinity optimization is a time-consuming process that does not always yield an appropriate candidate TCR. Time is of the essence for personalized therapies that target neoantigens from cancer patients (27). Methods for identification of cancer-reactive TCRs, although improving, still primarily rely on isolation of TCRs from tumor-reactive T-cell clones or TILs where the cancer antigen is used to screen or expand specific T cells. Notwithstanding the time-frame, the success rates of these approaches depend in part on the ability to clone and expand T cells or to isolate TILs with desired specificity. These rates vary among antigens and T-cell sources (49). Isolation of the validated antigen-specific α and β genes can also present a challenge (28,30).

We have suggested that the future of therapeutic TCR isolation will involve rapid and high throughput *de novo* approaches (31), analogous to what is now achievable for antibodies. Libraries with TCR diversity in the range of normal T-cell repertoires can be generated, with diversity focused on peptide-binding regions of a TCR (e.g. CDR3s). The advantage of this approach over antibody scaffolds is that TCR scaffolds have already been designed by evolution (using e.g. CDR2 loops; (39)) to dock over the pepMHC antigen such that maximal peptide specificity can be achieved. These *in vitro* methods can be adopted to generate potential therapeutic TCRs in a time scale closer to the demands of clinical application in patients, with multiplex capabilities to screen many antigens. Here we show that this approach can provide TCRs with the desired specificities necessary for adoptive T-cell therapies. We examined a TCR called RD1, generated by this approach against the cancer antigen MART-1/HLA-A2, and showed that it had all of the hallmarks of normal peptide fine specificity. RD1 also exhibited the affinity and specificity required for adoptive T-cell therapies involving recruitment of CD4⁺ and CD8⁺ T cells.

Although the RD1 TCR binds to MART-1/HLA-A2, the question remained whether it exhibited similar peptide fine specificity as conventional TCRs. To examine this, we compared an affinity-matured version of a conventional TCR called T1. We found that the fine specificities were almost identical, focusing the binding in both cases on positions P4 to P6 in the center of the peptide. Two other MART-1 binding TCRs, Mel5 and DMF5, also focus binding on the P4 to P6 region with the central GIG motif of MART-1/HLA-A2 (44) (45). The impact of the glycine residues suggests that conformational effects on the backbone provide additional opportunities for specificity. Like all class I MHC interacting peptides, functional specificity is also conferred by requirements for binding to MHC, such that to be functionally presented on the surface, the anchor positions are also restricted.

The peptide specificity for these TCRs contrasts with studies of a TCR-like antibody (ESK1) against the WT1/HLA-A2 complex (50). ESK1 exhibited less dependence on the peptide due to docking only at the N-terminus of the peptide/HLA-A2. This docking results in fewer interactions with the exposed peptide and greater cross-reactivity with other peptides. In contrast, a higher affinity TCR against WT1/HLA-A2 exhibited specific responses in CD8⁺ cells (34). Nevertheless, the ability of higher affinity TCRs to cross-react functionally with structurally similar peptides is due in part to the threshold for T-cell activation; raising the affinity against cognate antigens can yield cross-reactions that are functionally above the affinity threshold (51). On the other hand, a higher affinity TCR against NY-ESO-1/HLA-A2 with no cross-reactivity has been used in the clinic without apparent toxicity issues (52,53). Although we have focused here on analyzing the specificities of engineered, high affinity TCRs, there are also examples of low affinity, autoimmune TCRs that recognize many (10⁶) peptides (54).

We found that both T1 and RD1 were able to drive CD4⁺ T-cell activity, without apparent stimulation by endogenous peptide/HLA-A2 complexes. Nevertheless, in contrast to our expectations based on the convergence of the fine specificities of T1 and RD1, only the T1 TCR mediated CD8-dependent activity when stimulated in the absence of MART-1, using HLA-A2⁺ cells as APCs. The APCs included both mouse AAD⁺ spleen cells and human tumor lines T2 and HEK293F indicating that either mouse or human endogenous peptides can mediate the reactivity. Using the MART-1 peptide sequence in a restricted proteome scan, we identified a number of structurally similar self-peptides. One of these, TM74B-9, bound to HLA-A2 and to both RD1 and T1 TCRs. The binding to these TCRs required tetramers of the TM74B-9/HLA-A2 complex, indicating the affinity was low. The low affinity of this complex and perhaps other self-peptide complexes could explain why no basal CD4⁺ T-cell activity was detected in the presence of APC only (i.e. functional activity required CD8).

To further explore whether there are other self-peptides (in addition to TM74B-9) with structural similarity to MART-1 that stimulate CD8⁺ T cells with T1 but not RD1, we used a 'relaxed' proteome search based on binding signatures of multiple MART-1 binding TCRs (T1, RD1, Mel5 and DMF5) (44,45). We synthesized the top 36 peptides and tested them for stimulation of CD8⁺ T cells transduced with the T1 or RD1 TCRs. The T1 transduced CD8⁺ T cells were stimulated with 20 of the 36 self-peptides identified in this human proteome scan. In contrast, the RD1 TCR mediated activity with only one of the peptides. It is unclear if any of the peptides are processed and presented by HLA-A2 but the cross-reactivity with the T1 TCR likely accounts for the basal activity of APCs in the absence of the MART-1 peptide.

A previous report described a fatality in a clinical trial with adoptive T cells transduced with the MART-1-reactive TCR 1D3 (55). Although the 1D3 TCR was not affinity-enhanced, transduced CD4⁺ T cells bound to MART-1/HLA-A2 tetramer, suggesting that it exhibits an affinity higher than most cancer-reactive TCRs (6,7). It is unclear if 1D3-transduced CD8⁺ T cells showed reactivity with normal tissue. Given the reactivity of T1 and RD1 TCRs with the peptides identified in this study, it is possible that other MART-1-reactive TCRs will show similar cross-reactivity.

In conclusion, RD1 exhibits the features that are desirable for driving both CD4⁺ and CD8⁺ T cells' activity without reactivity against other endogenous peptide/HLA-A2 complexes. Like RD1 and the collection of affinity variants derived from it (43), the *de novo* isolation of TCRs from CDR libraries provides a platform to isolate TCRs with varying affinities against multiple cancer antigens. We envision isolation of panels of antigen-specific candidate TCRs from libraries generated on multiple TCR scaffolds, providing an advantage in throughput and time compared to conventional T-cell cloning and TCR identification. We are not suggesting that TCRs isolated by this means will always be less cross-reactive than TCRs isolated by conventional means. Rather, we argue that it should be possible to engineer and 'tune' both specificity and affinity of TCRs, regardless of their origin. In either case, adoptive T-cell applications will still require testing against structurally similar peptides from proteome scans, and screening against APCs and normal tissues with endogenous pep/HLA-A2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Krogsgaard M, Davis MM. How T cells 'see' antigen. *Nature immunology* 2005;6(3):239–45c. [PubMed: 15716973]
2. Arstila TP, Casrouge A, Baron V, Even J, Kanellopoulos J, Kourilsky P. A direct estimate of the human alphabeta T cell receptor diversity. *Science* 1999;286(5441):958–61c. [PubMed: 10542151]
3. Mason D A very high level of crossreactivity is an essential feature of the T- cell receptor. *Immunol Today* 1998;19(9):395–404c. [PubMed: 9745202]
4. Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. *Annu Rev Immunol* 2003;21:139–76c. [PubMed: 12414722]
5. Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, et al. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clin Cancer Res* 2009;15(17):5323–37c. [PubMed: 19723653]
6. Aleksic M, Liddy N, Molloy PE, Pumphrey N, Vuidepot A, Chang KM, et al. Different affinity windows for virus and cancer-specific T-cell receptors: implications for therapeutic strategies. *Eur J Immunol* 2012;42(12):3174–9c. [PubMed: 22949370]
7. Stone JD, Chervin AS, Kranz DM. T-cell receptor binding affinities and kinetics: impact on T-cell activity and specificity. *Immunology* 2009;126(2):165–76c. [PubMed: 19125887]
8. Holler PD, Kranz DM. Quantitative analysis of the contribution of TCR/pepMHC affinity and CD8 to T cell activation. *Immunity* 2003;18:255–64c. [PubMed: 12594952]
9. Holler PD, Holman PO, Shusta EV, O'Herrin S, Wittrup KD, Kranz DM. In vitro evolution of a T cell receptor with high affinity for peptide/MHC. *Proc Natl Acad Sci U S A* 2000;97(10):5387–92c. [PubMed: 10779548]

10. Li Y, Moysey R, Molloy PE, Vuidepot AL, Mahon T, Baston E, et al. Directed evolution of human T-cell receptors with picomolar affinities by phage display. *Nature biotechnology* 2005;23(3):349–54c.
11. Chervin AS, Aggen DH, Raseman JM, Kranz DM. Engineering higher affinity T cell receptors using a T cell display system. *Journal of immunological methods* 2008;339(2):175–84c. [PubMed: 18854190]
12. Malecek K, Zhong S, McGary K, Yu C, Huang K, Johnson LA, et al. Engineering improved T cell receptors using an alanine-scan guided T cell display selection system. *Journal of immunological methods* 2013;392(1-2):1–11c. [PubMed: 23500145]
13. Pierce BG, Hellman LM, Hossain M, Singh NK, Vander Kooi CW, Weng Z, et al. Computational design of the affinity and specificity of a therapeutic T cell receptor. *PLoS Comput Biol* 2014;10(2):e1003478c. [PubMed: 24550723]
14. Riley TP, Singh NK, Pierce BG, Baker BM, Weng Z. Computational Reprogramming of T Cell Antigen Receptor Binding Properties. *Methods in molecular biology* (Clifton, NJ) 2016;1414:305–18c.
15. Sherman LA, Hesse SV, Irwin MJ, La Face D, Peterson P. Selecting T cell receptors with high affinity for self-MHC by decreasing the contribution of CD8. *Science* 1992;258(5083):815–8c. [PubMed: 1439792]
16. Sykulev Y, Brunmark A, Tsomides TJ, Kageyama S, Jackson M, Peterson PA, et al. High-affinity reactions between antigen-specific T-cell receptors and peptides associated with allogeneic and syngeneic major histocompatibility complex class I proteins. *Proc Natl Acad Sci USA* 1994;91:11487–91c. [PubMed: 7972089]
17. Schmitt TM, Aggen DH, Ishida-Tsubota K, Ochsenreither S, Kranz DM, Greenberg PD. Generation of higher affinity T cell receptors by antigen-driven differentiation of progenitor T cells in vitro. *Nature biotechnology* 2017;35(12):1188–95c.
18. Bentzen AK, Such L, Jensen KK, Marquard AM, Jessen LE, Miller NJ, et al. T cell receptor fingerprinting enables in-depth characterization of the interactions governing recognition of peptide-MHC complexes. *Nature biotechnology* 2018c.
19. Holler PD, Chlewicki LK, Kranz DM. TCRs with high affinity for foreign pMHC show self-reactivity. *Nature immunology* 2003;4(1):55–62c. [PubMed: 12469116]
20. Linette GP, Stadtmayer EA, Maus MV, Rapoport AP, Levine BL, Emery L, et al. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. *Blood* 2013;122(6):863–71c. [PubMed: 23770775]
21. Morgan RA, Chinnasamy N, Abate-Daga D, Gros A, Robbins PF, Zheng Z, et al. Cancer Regression and Neurological Toxicity Following Anti-MAGE-A3 TCR Gene Therapy. *J Immunother* 2013;36(2):133–51c. [PubMed: 23377668]
22. Zhao Y, Bennett AD, Zheng Z, Wang QJ, Robbins PF, Yu LY, et al. High-affinity TCRs generated by phage display provide CD4+ T cells with the ability to recognize and kill tumor cell lines. *J Immunol* 2007;179(9):5845–54c. [PubMed: 17947658]
23. Quezada SA, Simpson TR, Peggs KS, Merghoub T, Vider J, Fan X, et al. Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *J Exp Med* 2010;207(3):637–50c. [PubMed: 20156971]
24. Xie Y, Akpınarlı A, Maris C, Hipkiss EL, Lane M, Kwon EK, et al. Naïve tumor-specific CD4(+) T cells differentiated in vivo eradicate established melanoma. *J Exp Med* 2010;207(3):651–67c. [PubMed: 20156973]
25. Liddy N, Bossi G, Adams KJ, Lissina A, Mahon TM, Hassan NJ, et al. Monoclonal TCR-redirected tumor cell killing. *Nat Med* 2012;18(6):980–7c. [PubMed: 22561687]
26. Garber K Driving T-cell immunotherapy to solid tumors. *Nature biotechnology* 2018;36(3):215–9c.
27. Stronen E, Toebes M, Kelderman S, van Buuren MM, Yang W, van Rooij N, et al. Targeting of cancer neoantigens with donor-derived T cell receptor repertoires. *Science* 2016;352(6291):1337–41c. [PubMed: 27198675]
28. Parkhurst M, Gros A, Pasetto A, Prickett T, Crystal JS, Robbins P, et al. Isolation of T-Cell Receptors Specifically Reactive with Mutated Tumor-Associated Antigens from Tumor-Infiltrating

- Lymphocytes Based on CD137 Expression. Clin Cancer Res 2017;23(10):2491–505c. [PubMed: 27827318]
29. Bethune MT, Li XH, Yu J, McLaughlin J, Cheng D, Mathis C, et al. Isolation and characterization of NY-ESO-1-specific T cell receptors restricted on various MHC molecules. Proc Natl Acad Sci U S A 2018;115(45):E10702–e11c. [PubMed: 30348802]
 30. Scheper W, Kelderman S, Fanchi LF, Linnemann C, Bendle G, de Rooij MAJ, et al. Low and variable tumor reactivity of the intratumoral TCR repertoire in human cancers. Nat Med 2018c.
 31. Smith SN, Wang Y, Baylon JL, Singh NK, Baker BM, Tajkhorshid E, et al. Changing the peptide specificity of a human T-cell receptor by directed evolution. Nat Commun 2014;5:5223c. [PubMed: 25376839]
 32. Smith SN, Sommermeyer D, Piepenbrink KH, Blevins SJ, Bernhard H, Uckert W, et al. Plasticity in the Contribution of T Cell Receptor Variable Region Residues to Binding of Peptide-HLA-A2 Complexes. J Mol Biol 2013;425(22):4496–507c. [PubMed: 23954306]
 33. Rodenko B, Toebes M, Hadrup SR, van Esch WJ, Molenaar AM, Schumacher TN, et al. Generation of peptide-MHC class I complexes through UV-mediated ligand exchange. Nat Protoc 2006;1(3):1120–32c. [PubMed: 17406393]
 34. Harris DT, Hager MV, Smith SN, Cai Q, Stone JD, Kruger P, et al. Comparison of T Cell Activities Mediated by Human TCRs and CARs That Use the Same Recognition Domains. J Immunol 2018;200(3):1088–100c. [PubMed: 29288199]
 35. Harris DT, Singh NK, Cai Q, Smith SN, Vander Kooi CW, Procko E, et al. An Engineered Switch in T Cell Receptor Specificity Leads to an Unusual but Functional Binding Geometry. Structure (London, England : 1993) 2016;24(7):1142–54c.
 36. Engels B, Cam H, Schuler T, Indraccolo S, Gladow M, Baum C, et al. Retroviral vectors for high-level transgene expression in T lymphocytes. Hum Gene Ther 2003;14(12):1155–68c. [PubMed: 12908967]
 37. Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA, Perarnau B. HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice. J Exp Med 1997;185(12):2043–51c. [PubMed: 9182675]
 38. Sharma P, Kranz DM. Subtle changes at the variable domain interface of the T-cell receptor can strongly increase affinity. J Biol Chem 2018;293(5):1820–34c. [PubMed: 29229779]
 39. Garcia KC, Adams JJ, Feng D, Ely LK. The molecular basis of TCR germline bias for MHC is surprisingly simple. Nature immunology 2009;10(2):143–7c. [PubMed: 19148199]
 40. Cole DK, Yuan F, Rizkallah PJ, Miles JJ, Gostick E, Price DA, et al. Germ line-governed recognition of a cancer epitope by an immunodominant human T-cell receptor. J Biol Chem 2009;284(40):27281–9c. [PubMed: 19605354]
 41. Borbulevych OY, Piepenbrink KH, Baker BM. Conformational melding permits a conserved binding geometry in TCR recognition of foreign and self molecular mimics. J Immunol 2011;186(5):2950–8c. [PubMed: 21282516]
 42. Sykulev Y, Joo M, Vturina I, Tsomides TJ, Eisen HN. Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. Immunity 1996;4:565–71c. [PubMed: 8673703]
 43. Harris DT, Wang N, Riley TP, Anderson SD, Singh NK, Procko E, et al. Deep mutational scans as a guide to engineering high-affinity T cell receptor interactions with peptide-bound MHC. J Biol Chem 2016c.
 44. Ekeruche-Makinde J, Clement M, Cole DK, Edwards ES, Ladell K, Miles JJ, et al. T-cell receptor-optimized peptide skewing of the T-cell repertoire can enhance antigen targeting. J Biol Chem 2012;287(44):37269–81c. [PubMed: 22952231]
 45. Gee MH, Han A, Lofgren SM, Beausang JF, Mendoza JL, Birnbaum ME, et al. Antigen Identification for Orphan T Cell Receptors Expressed on Tumor-Infiltrating Lymphocytes. Cell 2018;172(3):549–63.e16c. [PubMed: 29275860]
 46. Vigneron N, Stroobant V, Van den Eynde BJ, van der Bruggen P. Database of T cell-defined human tumor antigens: the 2013 update. Cancer immunity 2013;13:15c. [PubMed: 23882160]

47. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nature reviews Immunology* 2012;12(4):269–81c.
48. Stromnes IM, Schmitt TM, Chapuis AG, Hingorani SR, Greenberg PD. Re-adapting T cells for cancer therapy: from mouse models to clinical trials. *Immunol Rev* 2014;257(1):145–64c. [PubMed: 24329795]
49. Theaker SM, Rius C, Greenshields-Watson A, Lloyd A, Trimby A, Fuller A, et al. T-cell libraries allow simple parallel generation of multiple peptide-specific human T-cell clones. *Journal of immunological methods* 2016;430:43–50c. [PubMed: 26826277]
50. Ataie N, Xiang J, Cheng N, Brea EJ, Lu W, Scheinberg DA, et al. Structure of a TCR-Mimic Antibody with Target Predicts Pharmacogenetics. *J Mol Biol* 2016;428(1):194–205c. [PubMed: 26688548]
51. Stone JD, Kranz DM. Role of T cell receptor affinity in the efficacy and specificity of adoptive T cell therapies. *Front Immunol* 2013;4:244c. [PubMed: 23970885]
52. Robbins PF, Kassim SH, Tran TL, Crystal JS, Morgan RA, Feldman SA, et al. A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response. *Clin Cancer Res* 2015;21(5):1019–27c. [PubMed: 25538264]
53. Rapoport AP, Stadtmauer EA, Binder-Scholl GK, Goloubeva O, Vogl DT, Lacey SF, et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nat Med* 2015;21(8):914–21c. [PubMed: 26193344]
54. Wooldridge L, Ekeruche-Makinde J, van den Berg HA, Skowera A, Miles JJ, Tan MP, et al. A single autoimmune T cell receptor recognizes more than a million different peptides. *J Biol Chem* 2012;287(2):1168–77c. [PubMed: 22102287]
55. van den Berg JH, Gomez-Eerland R, van de Wiel B, Hulshoff L, van den Broek D, Bins A, et al. Case Report of a Fatal Serious Adverse Event Upon Administration of T Cells Transduced With a MART-1-specific T-cell Receptor. *Molecular therapy : the journal of the American Society of Gene Therapy* 2015;23(9):1541–50c. [PubMed: 25896248]

Figure 1: Two different approaches to isolate MART-1/HLA-A2 reactive T-cell receptors. The general methods used to isolate and engineer two TCRs, T1 (32) and RD1 (31), are shown.

Figure 2: Interaction of MART-1/HLA-A2 with T1 and RD1 TCR.
A, Structure of RD1-TCR with MART-1 HLA-A2 (PDB: 5E9D) is shown, with a focus on the binding interface. As shown, CDR3 loops of α and β chains of the TCR dock primarily over the peptide (35). **B**, Sequences of T1 and RD1 TCR at their CDRs. T1 TCR contains TRAV12-2 and TRBV14 chains, whereas the RD1 TCR contains TRAV12-2 and TRBV6-5. **C**, Contribution of various CDRs of T1 and RD1 TCR for binding to MART-1/HLA-A2 based on deep mutational scans (35,39). CDR: complementarity determining region; FR: framework.

Figure 3: Fine specificity of T1 and RD1 TCR for binding to MART-1/HLA-A2.

A, B, Yeast-displayed T1 or RD1 TCRs were stained with nine different concentrations of MART-1/HLA-A2 or its single-site variants (conserved amino acid and alanine substitutions in **A** and **B** respectively) ranging from 4 nM to 1 μ M. The dissociation constants (K_D) for their interaction were determined by fitting the binding data (Supplementary Fig. S1) to a 4-parameter regression model. In cases where the data fit the model poorly due to no or weak binding observed, K_D were shown as a lower limit of the assay and are indicated as *. **C,** An

overlay of MART-1 peptide from structures of various TCRs (Me15, DMF5, RD1-TCR) with MART-1/HLA-A2.

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Figure 4: Activity of T1 and RD1 TCR in primary CD4⁺ T cells.

A, Transduction efficiency of Mock, T1 or RD1-TCR transduced primary CD4⁺ T cells determined by staining with 10 nM MART-1/HLA-A2 tetramers. **B**, **C**, Activation of T1-TCR or mock (**B**) (or, RD1-TCR or mock (**C**)) transduced CD4⁺ T cells upon stimulation with various concentrations of peptides in the presence of HLA-A2/D^b splenocytes as antigen presenting cells. IFN γ in culture supernatants 24 hours after stimulation was measured by ELISA. Stimulation of T1-TCR or RD1-TCR transduced T cells in the presence of HLA-A2/D^b splenocytes only is indicated as (O) or “No peptide”.

of transduced cells under each condition was measured in duplicate. Similar results were obtained with T1-TCR transduced into CD4⁺ T cells from AAD⁺ mice in two independent experiments. Stimulation of RD1 TCR was measured in one experiment.

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Figure 5: Activity of T1 and RD1 TCR in primary CD8⁺ T cells.

A, Transduction efficiency of Mock, T1 or RD1-TCR transduced primary CD8⁺ T cells determined by staining with 10 nM MART-1/HLA-A2 tetramers. **B**, **C**, Activation of T1-TCR or mock (**B**) (or, RD1-TCR or mock) (**C**) transduced CD8⁺ T cells upon stimulation with various concentrations of peptides in the presence of HLA-A2/D^b splenocytes as antigen presenting cells. IFN γ in culture supernatants 24 hours after stimulation was measured by ELISA. Stimulation of T1-TCR or RD1-TCR transduced T cells in the presence of HLA-A2/D^b splenocytes only is indicated as (O) or “No peptide”. Stimulation

of transduced cells under each condition was measured in duplicate. Similar results were obtained with T1-TCR transduced into CD8⁺ T cells from AAD⁺ mice in an independent experiment. Stimulation of RD1 TCR was measured in one experiment.

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Figure 6: Reactivity of T1 or RD1 TCR transduced CD8⁺ T cells with human cell lines. **A**, HLA-A2 expression on four human cell lines, and AAD splenocytes from HLA-A2/D^b transgenic C57BL/6 mice was assessed by staining with FITC conjugated anti-human HLA-2 antibody (clone BB7.2). The levels of HLA-A2 expression on all cell lines were validated in two independent experiments. **B**, **C**, **D**, **E**, Activation of T1-TCR, RD1-TCR or mock transduced CD8⁺ T cells upon stimulation with T2 (**B**), HEK293F (**C**), MCF7 (**D**) or Jurkat (**E**) as APCs at 37°C, 5%CO₂, in the absence of exogenously added peptides. IFN γ in culture supernatants 24 hours after stimulation was measured by ELISA. Stimulation of transduced cells in the absence of peptides was measured in duplicate in two independent experiments.

Figure 7:

Reactivity of T1 or RD1 TCR transduced CD8⁺ T cells with human peptides containing central “GIG” motif.

T1-TCR, RD1-TCR or mock-transduced CD8⁺ T cells were stimulated with 1 μ M peptide using T2 cells as APCs at amounts that had reduced basal levels of stimulation. IFN γ in culture supernatants 24 hours after stimulation was measured by ELISA. Each TCR was stimulated with 1 μ M of each peptide in duplicate. Similar results were obtained when 100-fold higher peptide concentration was used to stimulate the TCR transduced cells in an additional experiment (Sup Fig S3). Stimulation of T1 and RD1 TCR transduced CD8⁺ T cells with MART-1 and TM74B-9 was measured in a total of four independent experiments.