T-cell Receptors Engineered De Novo for Peptide Specificity Can Mediate Optimal T-cell Activity without Self Cross-Reactivity

Preeti Sharma, Daniel T. Harris, Jennifer D. Stone, and David M. Kranz

Abstract

Despite progress in adoptive T-cell therapies, the identification of targets remains a challenge. Although chimeric antigen receptors recognize cell-surface antigens, T-cell receptors (TCR) have the advantage that they can target the array of intracellular proteins by binding to peptides associated with major histocompatibility complex (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 TCRβ 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 such as RD1, generated de novo against cancer antigens, can serve as an alternative to TCRs generated from T-cell clones.

Introduction

T-cell receptors (CD8 TCR) expressed on T cells recognize diverse self-, foreign-, and mutated peptides presented by proteins encoded by genes of the major histocompatibility complex (MHC; ref. 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 submicro-molar 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 that 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 the 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., refs. 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: (i) stimulation of PBMCs with peptide antigen or isolation of tumor-infiltrating lymphocytes (TIL) from tumors, (ii) in vitro expansion of T cells and assessment of peptide specificity, (iii) isolation of TCR α and β genes from T-cell clones or single-cell PCR, (iv) transfer of the candidate TCR genes into T cells for verification of specificity with the peptide antigen, (v) TCR-affinity engineering by in vitro techniques, or screens of many TCRs, for optimal affinity/activity (e.g., refs. 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 PBMCs with peptide antigen or isolation of tumor-infiltrating lymphocytes (TIL) from tumors, (ii) in vitro expansion of T cells and assessment of peptide specificity, (iii) isolation of TCR α and β genes from T-cell clones or single-cell PCR, (iv) transfer of the candidate TCR genes into T cells for verification of specificity with the peptide antigen, (v) TCR-affinity engineering by in vitro techniques, or screens of many TCRs, for optimal affinity/activity (e.g., refs. 27–30).

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T-cell isolation and transduction with TCR
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 10⁶ cells per well in complete DMEM (with glutamax 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 μg plasmid DNA or mock (no DNA) in the presence of Lipofectamine 2000 (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-μm 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 IL2 (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 IL2 (Invitrogen). After 4 hours, cells were washed and cultured in complete IMDM media. After 48 hours, retroviral infected mononuclear cells were harvested and filtered through 0.45-μm 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 IL2 (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 IL2, 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/D² transgenic C57BL/6 mice (AAD⁺ mice, expressing the α1 and α2 domain of HLA-A2, fused with the β3 domain of H-2D³; ref. 37), or human cell lines (T2, HEK293F, MCF7, and Jurkat) were used as antigen-presenting cells (APC). 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 physiologic 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+; 55,000–75,000) 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 (IFNγ) using an ELISA (Invitrogen) following the manufacturer's instructions.

### Results

**Comparison of two TCRs generated by distinct approaches**

Here, we compared two TCRs, T1-S18.45 and RD1-MART1HIGH, that bind to the same cancer antigen, MART-1/HLA-A2 (Fig. 1). Here onward, 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:MART1/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 submicromolar affinities using standard CDR mutagenesis followed by yeast display and selection (KD of 100 ± 5 nmol/L, and 500 ± 5 nmol/L, for T1 and RD1, respectively; refs. 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 CDR3a and CDR3b 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; ref. 35). Side-by-side analysis of the average reduction in prevalence for substitutions among residues in each CDR is 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). Because CDR3 loops of TCRs dock predominantly over the peptide in
TCR:pepMHC structures (Fig. 2A, inset; ref. 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 and 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 (Mel5 or DMF5) (Fig. 3C; refs. 35, 40, 41). Changing residues at the N-terminus (E1, L2, and A3) as well as C-terminus (T9 and V10) to either conserved amino acids or alanine did not significantly affect the binding affinity of either TCR (Fig. 3A and B), although substitutions of these residues could, of course, affect 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 and 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 submicromolar 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 TCRs.
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 spleenocytes from HLA-A2/Db transgenic mice. These 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-2Db.

T1-TCR\(^-\) and RD1-TCR\(^-\) transduced CD4\(^+\) T cells were both activated (EC\(_{50}\)/C\(_{24}\) 10 nmol/L) by MART-1 peptide (Fig. 4B and 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 with 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\(\gamma\) 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 (EC\(_{50}\) \(\sim\) 1 nmol/L; 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), 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 and C). The extent of activation was correlated with the amount of HLA-A2, with MCF7 and Jurkat showing no stimulatory ability (Fig. 6D and E). In contrast to T1, RD1-transduced CD8\(^+\) T cells were not stimulated by T2 or HEK293F cells (Fig. 6B and C), consistent with the observation using AAD\(^+\) APCs.
determined by staining with 10 nmol/L MART-1/HLA-A2 tetramers.

**Figure 4.** Activity of T1 and RD1 TCRs in primary CD4+ T cells. A, Transduction efficiency of mock-, Th-, or RD1-TCR-transduced primary CD4+ T cells determined by staining with 10 nmol/L MART-I/HLA-A2 tetramers. B and C, Activation of T1-TCR– or mock-transduced (B) or RD1-TCR– or mock-transduced (C) CD4+ T cells upon stimulation with various concentrations of peptides in the presence of HLA-A2/D9 splenocytes as APCs. IFNγ 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/D9 splenocytes only is indicated as (Δ) or "No peptide." Stimulation 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.

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 the UniProtKB database) based on similar binding signatures of the two TCRs from our experimental data (Fig. 3A and 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][AST][AV] for the nonamer scan or [ADE][AIL][AG][AGS][IL][AGS][AIL][AST][AV] for the decamer scan). These “tight” scans yielded 46 matches, with 19 unique sequences (excluding MART-1) in the human proteome. Because HLA-A2/D9 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 the 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 (Kd, predicted = 2,254 nmol/L): a peptide from Transmembrane protein 74B (TM74B-8a; IAGLGLLTV) and a peptide from RNA- binding protein Nova-1 (ALGSLAAATA) with predicted Kd values of 2,036 and 1,772 nmol/L, respectively. TM74B-9 was most similar to MART-1 nonamer (AAGIGILTV) as it was identical in six of nine positions and homologous in two 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.iiab.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 tetrmers, to T1 and RD1 TCRs (Supplementary Fig. S2). In addition, we also evaluated binding of the TM74B peptide variants to higher affinity mutants of the T1 and RD1 TCRs (T1-TCRIGH, RD1-TCRHIGH) that were engineered previously (refs. 38, 43; Supplementary Fig. S2). This allowed us to compare the binding of HLA-A2 complexes of the TM74B variant peptides and MART-1 to a panel of TCRs that ranged in affinity for MART-1/HLA-A2 from 2 to 500 nmol/L. Binding of T1 to 1 μmol/L 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 tetrmers was higher (Supplementary Fig. S2B). This result indicates that both TCRs bound to the TM74B nonamer but with low affinity. Although both T1-TCRIGH and RD1-TCRHIGH exhibited similar dissociation constants for MART-1/HLA-A2 (Kd = 2 nmol/L; refs. 38, 43), binding to TM74B-8b/HLA-A2 and TM74B-9/HLA-A2 as monomers was only detected with T1-TCRIGH but not with RD1-TCRHIGH (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. 3A and 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 (Mel5 and DMF5; refs. 44, 45) were also included in this string design, thus yielding [XI][M,I][G,][G][AFILMSWV- [AGIKLMNVWY][ADIKLMNPQRTSV][LIVMA] and [DEFGHKML- MNS][LMI][AEFGKLPSTWV][G][G][AFILMSWV][AGIKLMNV- WY][ADIKLMNPQRTSV][LIVMA] as 9-mer and 10-mer strings.
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 the 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 the identification of cancer-reactive TCRs, although improving, still primarily rely on the 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; ref. 39) to dock to 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 specificity and that the RD1 TCR represents a preferred alternative to the T1 TCR, based on its antigen specificity and reduced cross-reactivity.

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 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 μmol/L), whereas RD1 was stimulated by only 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.
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 (10\(^6\)) 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\(^{+}\) T-cell 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; refs. 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-cell 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 with 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.

Disclosure of Potential Conflicts of Interest

D.M. Kranz has ownership interest (including patents) in Bellicum Pharmaceuticals, Agenus Inc., and Jounce Therapeutics and is a consultant/advisory board member for AbbVie. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: P. Sharma, D.T. Harris, D.M. Kranz
Development of methodology: P. Sharma, D.T. Harris, J.D. Stone
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Sharma, D.M. Kranz
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Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): P. Sharma, D.T. Harris, D.M. Kranz

Writing, review, and/or revision of the manuscript: P. Sharma, D.T. Harris, J.D. Stone, D.M. Kranz

Study supervision: P. Sharma, D.M. Kranz

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De Novo T-cell Receptors Engineered
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