Subtle changes at the variable domain interface of the T-cell receptor can strongly increase affinity

Received for publication, August 25, 2017, and in revised form, December 3, 2017 Published, Papers in Press, December 11, 2017, DOI 10.1074/jbc.M117.814152

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Most affinity-maturation campaigns for antibodies and T-cell receptors (TCRs) operate on the residues at the binding site, located within the loops known as complementarity-determining regions (CDRs). Accordingly, mutations in contact residues, or so-called “second shell” residues, that increase affinity are typically identified by directed evolution involving combinatorial libraries. To determine the impact of residues located at a distance from the binding site, here we used single-codon libraries of both CDR and non-CDR residues to generate a deep mutational scan of a human TCR against the cancer antigen MART-1/HLA-A2. Non-CDR residues included those at the interface of the TCR variable domains (Vα and Vβ) and surface-exposed framework residues. Mutational analyses showed that both Vα/Vβ interface and CDR residues were important in maintaining binding to MART-1/HLA-A2, probably due to either structural requirements for proper Vα/Vβ association or direct contact with the ligand. More surprisingly, many Vα/Vβ interface substitutions yielded improved binding to MART-1/HLA-A2. To further explore this finding, we constructed interface libraries and selected them for improved stability or affinity. Among the variants identified, one conservative substitution (F45L) was most prevalent. Further analysis of F45L showed that it enhanced thermostability and increased affinity by 60-fold. Thus, introducing a single hydroxyl group at the Vα/Vβ interface, at a significant distance from the TCR-peptide-MHC binding site, remarkably affected ligand binding. The variant retained a high degree of specificity for MART-1/HLA-A2, indicating that our approach provides a general strategy for engineering improvements in either soluble or cell-based TCRs for therapeutic purposes.

The two major classes of antigen receptors in the immune system, antibodies and T-cell receptors (TCRs),2 are heterodimers that recognize their antigens through the action of six hypervariable loops called complementarity-determining regions (CDRs). Three of the CDRs are encoded by the variable region domain of one chain, and three of the CDRs are encoded by the variable region domain of the other chain. The affinity of antibodies can be increased naturally by B cells through a process of somatic hypermutation (1–3) that focuses on the V regions. In contrast, T cells do not contain the machinery for hypermutation, and their receptors are thus of low affinity (4–6).

In the case of antibodies and TCRs, it is now possible to affinity-mature their binding sites in vitro using the process of directed evolution (2, 7, 8). This process has typically involved the generation and selection of large libraries of CDR mutants in a system such as phage, ribosome, or yeast display. In the early 1990s, Winter and colleagues (9, 10) displayed naive as well as synthetic human antibody repertoires on the surface of phage that could be selected with different antigens to obtain desired antigen specificities and to use in vitro affinity maturation of antibodies with affinities in the low nanomolar range (11). In the same decade, ribosome, phage, and yeast display were also developed to improve the affinity of antibodies (12–15). At the same time, the yeast-display system was used to engineer stabilized single-chain mutants of the TCR 2C (16) and subsequently to isolate higher affinity mutants of the 2C TCR by generating libraries in CDR residues, followed by selection with the peptide-major histocompatibility complex (pep-MHC) ligands (17–19). Other TCRs have been engineered for high affinity against their pep-MHC ligands (20–23), and more recently we created new specificities using directed evolution of the human TCR A6 (24).

An additional challenge in the field of antibody and soluble TCR engineering has been to improve their stability and yields for use in therapeutic applications. One approach to this problem has been to design mutations that promote preferential pairing of antibody heavy and light chains, or TCR α and β chains. For example, the “knobs in hole” concept of Carter and colleagues (25–27) has been used to generate more stable antibodies or to drive the pairing of the appropriate chains in the production of bispecific antibodies. Similarly, two cytokines have been engineered into the constant regions of the TCR α and β chains to stabilize the extracellular domains in soluble form or as a cell-surface heterodimer (28, 29). In addition to directed evolution as a strategy to improve binding affinity, we

This work was supported by National Institutes of Health Grants CA178844 and CA187592 (to D. M. K.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains Figs. S1–S5.

1 The abbreviations used are: TCR, T-cell receptor; Vα, variable region of α chain of TCR; Vβ, variable region of β chain of TCR; CDR, complementarity-determining region; FR, framework region; scTCR, single chain T-cell receptor; pep-MHC, peptide complexed with major histocompatibility complex; HLA, human leukocyte antigen (refers to human major histocompatibility complex alleles); Kd, dissociation constant; Tm, melting temperature; DSF, differential scanning fluorimetry; SPR, surface plasmon resonance;

SCL, single-codon library; PDB, Protein Data Bank; PE, phycoerythrin; PCL, paired-codon library; β2m, β2–microglobulin; MFU, mean fluorescence units.

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have used the method to generate improved stability of TCR variable domains by selection of libraries of mutants throughout the V region using *Escherichia coli* mutator strains or error-prone PCR (16, 22, 30–34). Selection for stabilizing mutations was made possible by the observation that higher stability was associated with higher levels of display on yeast (16, 30). Mutations engineered through this process can act in multiple different mechanisms (e.g., through substitutions of exposed surface residues or the interface residues between V regions) (16, 30, 35). The strategies that have allowed for selection of stable variants of a protein have often used increasing temperatures as a selection pressure in the screening of yeast libraries for thermostable variants (36).

More recently, we used deep mutational scans (37–39) of TCRs to understand the role of CDR residues in binding and stability (40, 41). Single-codon libraries (SCLs) in all of the CDRs in TCRs against the viral antigen Tax−HLA-A2 and the cancer antigen MART-1−HLA-A2 were generated, and selections with peptide−HLA-A2 revealed the sequence fitness landscape for these TCRs (40). In addition, the approach showed that ~8% of the CDR substitutions yielded enhanced binding or stability. The combination of CDR mutations that individually yielded modest improvements in binding allowed the identification of TCR mutants that were 100-fold higher affinity, on par with directed evolution strategies (41).

Using both deep mutational scans and directed evolution, here we examined another human TCR against MART-1−HLA-A2, called T1, with a goal of determining whether V region interfaces could be engineered for greater stability and expression properties. Single-codon libraries were generated in both CDR and non-CDR residues, including framework residues located at the Va/Vβ interface, and the yeast-displayed libraries were selected with the antigen. Unexpectedly, numerous mutants were identified from the sequence fitness landscape where a substitution at the Va/Vβ interface yielded enrichment, similar to the number found in CDRs, a finding that could be due to higher yeast surface levels (e.g., due to improved stability) or higher affinity.

Based on these results and the known structures of TCRs, we generated dual-codon libraries of opposing pairs of interface residues and subjected these and the single-codon libraries to antigen selection by high-speed flow sorting. Independent isolates identified from these selections revealed a predominant interface mutant, F45BY. Remarkably, this subtle substitution not only showed enhanced surface display but exhibited a 50-fold improvement in affinity ($K_D = 2$ nM) in yeast-display experiments. The soluble, refolded TCR also exhibited a 60-fold increase in affinity and an increase in thermal stability. The F45BY mutant retained specificity for MART-1−HLA-A2, as it did not bind to other peptide−HLA-A2 complexes. These results demonstrate that subtle changes at the interface that are outside the antigen-binding site can operate at a distance to modulate binding site residues. In addition, they suggest that Va/Vβ interface substitutions should be included in efforts to identify TCRs with affinity and stability improvements for use as soluble therapeutics or in adoptive T-cell therapies.

**Results**

**Deep mutational scan of a single-chain (Vβ-linker-Vα) human TCR**

A TCR isolated from the human T-cell clone called INR1-T1, specific for MART-1−HLA-A2, was engineered previously as a single-chain TCR (scTCR) (Vβ16-linker-Vα2; TRBV30-linker-TRAV12-2 in IMGT nomenclature) by identifying mutations that enabled yeast surface display and expression as a soluble protein (23). This TCR is referred to as T1-WT here (Fig. S1). Sorting CDR libraries of the T1-WT scTCR yielded a mutant called T1-S18.45 that exhibited 710-fold higher affinity ($K_D = 45$ nM) than the wildtype affinity ($K_D = 32$ μM) (23). Because the T1-S18.45 mutant TCR (called T1 from here on) has sufficient affinity to perform binding selections by yeast display, it was used to conduct a deep mutational scan of all CDR residues and many framework residues by generating SCLs (Fig. S1). The framework residues focused in particular on those located at the interface of Va and Vβ domains so that we could determine whether the wildtype residues have been optimally selected, presumably by evolution or by processes involved in thymic development, for the interaction of the two domains. The issue for Vβ domain associations as dimers is even more complicated, as there are models suggesting that during thymic development, the Vβ domain of the rearranged β chain in the pre-TCR could interact either with the invariant α chain (pTα) or with residues in a second Vβ to form a dimer (42).

The SCL included 45 residues in the Va and 42 residues in the Vβ (Fig. 1 and Fig. S1). Of the 87 positions, 43 were located in the predicted CDRs (21 residues in the Va and 22 residues in Vβ). To determine which residues are likely to be located at the Va/Vβ interface, we relied on the structures of known TCRs, in particular four TCRs that all contained the same Va region (TRAV12-2, also known as Va2: Mel5 (pdb code 3HG1), DMF5 (pdb code 3QDG), RD1-MART1$^{\text{HIGH}}$ (pdb code 5E9D), and A6 (pdb code 1AO7) (40, 43–45). This analysis identified 15 residues at the interface in the Va and 16 residues at the interface in the Vβ. As an additional control, we constructed 13 SCLs in residues of surface framework regions (surface FR) that were predicted to be in surface-exposed positions but were distal to the pep-MHC-binding site (Fig. 1 and Fig. S1).
SCLs were constructed as described previously (41), and pooled libraries were transformed into yeast cells. The libraries were analyzed in comparison with the parental TCR T1 for binding to monomeric MART-1-HLA-A2 and tetrameric MART-1-HLA-A2 (Fig. 2). As expected, compared with the parental T1 TCR, the pooled SCL exhibited a reduction in binding indicative of a significant fraction of substitutions that lowered or eliminated binding to the ligands.

Based on the binding curves for the SCL and our previous experience with yeast selections in deep mutational scans, we chose to sort the libraries with 10 nM monomeric ligand (MART-1-HLA-A2), a concentration that was ~5-fold lower than the $K_D$ determined previously by surface plasmon resonance (23). We collected the top 1% of yeast cells with bound ligand, expanded them, isolated plasmid DNA, and subjected it to deep sequencing. DNA from the unselected SCL was also subjected to deep sequencing, and enrichment ratios for every substitution at each targeted residue were calculated relative to the unselected libraries and plotted on a log$_2$ scale. The analysis allowed the generation of a sequence fitness landscape with a color-coded scale (24).

![Figure 2. Binding titration of T1 TCR or SCLs with monomeric or tetrameric MART-1-HLA-A2 ligand.](image)

**T-cell receptor interface residues**

In addition to ligand binding, are all sensitive to selection with the MART1-HLA-A2 probe.

To gain a more quantitative assessment of the impact of individual CDR loops or framework regions, we averaged the enrichment values for every substitution within the six CDRs, the two interface regions, or the two surface-exposed framework regions (Fig. 4A, red bars). The results for the surface FR opposite the binding site represent the basal impact of substitutions on the folding, aggregation, and stability of the single-chain TCR, all involved in export and display on the surface. For example, changes of surface hydrophilic residues to hydrophobic residues would be expected to have a negative impact. Hence, substitutions of the surface FR regions show a significant impact on selection but exhibit the lowest average enhancement values compared with the other regions (see red bars in Fig. 4A). Conversely, residues in CDR3 in T1, followed by CDR3β, had the overall greatest impact of substitutions, suggesting that they play a key role in MART-1-HLA-A2 binding (Fig. 4A).

As previously noted in our deep mutational scans of two other TCRs (40, 41), a significant number of CDR substitutions were enriched (i.e. blue) in T1 relative to the wildtype amino acids (Figs. 3 and 4B). For example, transcripts with the following substitutions were enriched 8-fold or more compared with wildtype: D26eP, G28aS/H/F/Y/M, S31aG/A, S51aG/A, S91aA, I26βE/L, S27βP, H29βM, D30βE, N31βH, K51βQ, S53βW/L, and A97βT. Although CDR3α and CDR3β are positioned directly above the peptide in TCR-pep-MHC structures, enriched substitutions in T1 were distributed across all CDRs, indicating that they may act to optimize the interaction with MART-1-HLA-A2 either by direct contact or by inducing conformational changes that configure the contact residues in more optimal positions. Although we have observed such enhancing substitutions in the CDRs of a different TCR previously (41), we were particularly surprised that the Va/Vβ interface also contained numerous substitutions that were enriched (Figs. 3 and 4B) and in fact yielded a higher relative number of such substitutions when compared with the CDRs. For example, transcripts with the following substitutions in the interface were enriched 8-fold or more compared with wildtype: F33aS/N/R/W/M, M97aQ/K, D100aN/Q/K/R/V/I/L/M, M39βN, K41βT/N, E42βP, F45βY, E102βH/R/I, and Y104βV/I. Interestingly, Asp$^{196\text{c}}$ in T1 corresponds to the variable position ($X$) of the conserved FGXG motif that exists in J regions of $\alpha$ and $\beta$ domains of TCR and the $\kappa$ light chain of antibodies. In fact, in
the T1 TCR, it appears that X can be favorably substituted by almost any amino acid (Fig. 3). The enrichment observed for many interface substitutions suggests that there are significant opportunities to engineer more stable Vα/Vβ interactions by directed evolution.

Stability scans of the TCR single-codon libraries

As indicated, enrichment of mutants with a particular substitution in yeast-display selections of single-codon libraries can be due to an increase in stability (surface level) or affinity of the mutant (41). Surface levels of expressed proteins have been associated with the stability of the properly folded protein (30, 31, 46). To understand whether enrichment of mutants with some of the substitutions might have been due to increased stability, we decided to conduct several additional screens, followed by deep sequencing. First, we used a relatively high concentration of tetrameric MART-1-HLA-A2 (tetramer) (100 nM) that would in principle be more sensitive to surface levels than to the intrinsic affinity of the TCR (albeit still dependent on binding). In addition, we used the tetramer to select SCLs that were exposed to a higher temperature (45 °C for 30 min), thus enriching for the most stable TCRs (31). Finally, we used an anti-Vβ16 monoclonal antibody, which should not require a Vα-specific epitope but does require proper conformation of the protein because the antibody binds to native Vβ16+ TCRs on the cell surface.

Sequence fitness landscapes for each selection are shown in Figs. S2 and S3 and Fig. 5. The overall pattern of enrichment of residues in tetramer-selected SCLs, whether exposed to the

![Sequence fitness landscape of T1 TCR based on selection of single-codon libraries with monomeric MART-1-HLA-A2.](image-url)
higher temperature or not, was almost identical (Figs. S2 and S3). The identical profiles were most likely due to the already relatively high thermostability of the template T1 (see below (Fig. 9, D and E)), in part based on the stability of the Vα2 domain (47). The similarity in pattern of enrichment between the two selections was most apparent when plotting the enrichment values for each substitution selected with tetramer versus the enrichment value for the same substitution selected with “heat + tetramer” condition. Our previous deep mutational scans have shown that duplicate selection with the same ligands yield correlation ($R^2$) values that are $>0.8$ in the positive enrichment substitutions (blue) and correlations in the range of 0.5 in the negative enrichment substitutions (orange) (40). Overall correlation plots for the tetramer selections yielded $R^2$ values in the range of 0.45–0.65 (Fig. 6A), indicative of good correlations and substantiating the reproducibility of the deep mutational scan method (40). Whereas substitutions in CDRs, interface, and surface FR of Vα demonstrated good correlation (i.e. enriched or depleted to similar levels), substitutions in surface FR of Vβ exhibited poorer correlation in part due to their relatively smaller sample size and the lower level of their negative enrichment values.

The patterns of enrichment for the monomer selection (Fig. 3) and tetramer selections (Figs. S2 and S3) were also similar, although in general, the magnitude of enrichment values was overall reduced in the tetramer data set (Fig. 4A), leading to lower average correlation values between the two selection schemes ($R^2$ in the range 0.23–0.46) (Fig. 6B). Because both monomeric and tetrameric MART-1-HLA-A2 interact with the same binding site on the TCR, the differences are probably due to the impact of surface levels and hence stability. Nevertheless, the hierarchy of enhancement values for each CDR and framework region remained the same with the two selections (Fig. 6B). Interestingly, although most surface framework residues and many CDR residues appeared to have reduced impact compared with the monomer selection (i.e. values closer to 0), many interface substitutions retained their level of impact on enrichment or depletion. Accordingly, scatter plots indicated that there were typically higher correlations between enrichment/depletion of substitutions in interface residues (Fig. 6B) than in CDR residues. Whereas residues in surface FR of Vα were enriched or depleted to similar levels ($R^2 = 0.46$), substitutions in certain surface FR of Vβ were preferentially enriched with monomer selection compared with tetramer, hence leading to lower correlation ($R^2 = 0.09$). As mentioned above, these substitutions may have allowed for improved stability of Vβ and hence expression of the TCR on yeast surface rather than having an impact on binding affinity.

Selection with the anti-Vβ16 antibody (Fig. 5) provides information about the TCR using a probe that is completely different from the MART-1-HLA-A2 ligand (Fig. 3 and Figs. S2 and S3). Although the specific epitope of this antibody has not been identified, it binds only to T cells with a Vβ16+ TCR, and hence it binds to epitopes found on all Vβ16 domains (48). This excludes epitopes on Vα and those generated by the CDR3β loop, both of which vary among Vβ16+ T cells. Consistent with these expectations, examination of the anti-Vβ16 sequence fitness landscape (Fig. 5) showed overall less impact of Vα CDRs and Vα surface FR residues compared with monomer or tetramer selections (Fig. 4A). However, the Vβ CDRs also appeared to show less impact on anti-Vβ16 antibody binding than the MART-1-HLA-A2 ligand, suggesting that the epitope does not reside in the CDR1β or CDR2β. In contrast, substitutions of the surface-exposed residues of the Vβ framework (Fig. 1) were substantially depleted with the anti-Vβ16 antibody in contrast to selections with the MART-1-HLA-A2 ligand (Figs. 3B, 4A, and 5B). This finding was consistent with the lower correlation in this region between the selections with different ligands ($R^2 = 0.1$ in Fig. 6C and Fig. S4). We propose that this observation is consistent with this region comprising a part of the anti-Vβ16 epitope (Fig. S5). This possibility is supported by analysis of all human Vβ sequences, showing that this stretch of amino acids (GGMPSG) is unique to Vβ16 among all 60 Vβ genes.
Unexpectedly, the anti-Vβ16 probe showed that Vα and Vβ interface residues exhibited a sequence fitness landscape that was similar to that observed in the screens with the MART-1-HLA-A2 monomer and tetramer ligands (Figs. 3, 5, and 6C and Fig. 54). This result suggests that although the epitope does not reside in Vα, these Vα and Vβ interface residues are necessary for proper conformation of the Vβ domain. This observation is also consistent with the finding that stop codons in the Vα region were negatively selected, although the protein is cloned as a Vβ-linker-Vα construct. Screens of TCRs with different Vβ in yeast display using other anti-Vβ antibodies have shown no need for the Vα domain in the yeast-display format, or even enhanced surface levels when a stop codon was introduced into the Vα (32, 34, 49, 50). It is possible that the finding here with the anti-Vβ16 antibody reflects the unique structural requirements for the epitope of this antibody, compared with the other anti-Vβ antibodies. When comparing enrichment or depletion of substitutions in CDR residues when T1-SCL was selected with two different ligands (monomeric or tetrameric MART-1-HLA-A2 versus anti-Vβ16), lower $R^2$ values ($R^2 = 0.1–0.2$) indicated the requirement of unique substitutions for interacting with each ligand as expected (Fig. 6C and Fig. 54). However, as indicated, substitutions in interface residues exhibited better correlations ($R^2 = 0.2–0.5$).

**Interface libraries guided by sequence fitness landscapes lead to isolation of stable, high-affinity mutants**

To further assess the extent to which Vα/Vβ interface residues could impact stability and/or pep-MHC affinity, we took a directed evolution approach that used existing SCLs in inter-
face residues (Phe$^{33\alpha}$, Lys$^{41\alpha}$, Ser$^{42\alpha}$, Met$^{97\alpha}$, Asp$^{100\alpha}$, Met$^{39\beta}$, Lys$^{41\beta}$, Glu$^{42\beta}$, Lys$^{44\beta}$, Phe$^{45\beta}$, Met$^{60\beta}$, Phe$^{91\beta}$, Val$^{101\beta}$, Glu$^{102\beta}$, and Gly$^{106\beta}$) and additional, paired-codon libraries (PCLs) generated in interface residues. The choice of positions to randomize at the $\alpha$/V$\beta$ interface was based on the enrichment results of the deep mutational scan, together with an analysis of the structures of known TCRs (40, 43–45) to identify possible paired residues across the $\alpha$/V$\beta$ interface. For the latter, each interface residue was used to search for a possible binding partner in the opposing domain (within a 4-Å space). Accordingly, PCLs (Fig. S1) were constructed using PCR splicing by overlap extension in the following residues: Lys$^{41\beta}$–Asp$^{100\alpha}$, Glu$^{42\beta}$–Asp$^{100\alpha}$, Lys$^{44\beta}$–Met$^{97\alpha}$, Phe$^{45\beta}$–Met$^{97\alpha}$, Phe$^{91\beta}$–Lys$^{41\alpha}$, Phe$^{91\beta}$–Ser$^{42\alpha}$, and Glu$^{106\beta}$–Ser$^{42\alpha}$. A mixture of the SCLs and PCLs (referred to as interface libraries hereafter) were sorted twice with 1 nM monomeric MART-1$^{18528}$HLA-A2. To be able to screen for improved properties that would allow application of engineered TCRs in adoptive T-cell therapies or as soluble therapeutics in a physiologic setting, we included a sort in which yeast cells were incubated in 100% human serum. For example, exceptional stability in serum could enrich for substitutions that resist proteolysis or other serum-derived enzymes that lead to a reduction in surface levels of the TCRs. In this regard, incubation of the parental T1 TCR in serum yielded reduced staining with MART-1$^{18528}$HLA-A2 monomer (Fig. 7A).

A decrease in binding of the interface libraries to 10 nM MART-1$^{18528}$HLA-A2, compared to parental T1 TCR, was observed (Fig. 7, A and B). This was expected because most $\alpha$/V$\beta$ interface mutations destabilize the TCR (Fig. 2). The binding was reduced further when analyzed in the presence of serum. However, after two sorts in the presence or absence of serum, the interface libraries exhibited a strong increase in binding to MART-1$^{18528}$HLA-A2 (Fig. 7, C and D). The populations from both selections were able to bind MART-1$^{18528}$HLA-A2 at concentrations as low as 100 pM, significantly improved compared with the parental T1 TCR. For example, staining of the sorted population showed higher signal at 100 pM ligand (Fig. 7, C and D) than staining of the parental T1 at 10 nM ligand, a 100-fold higher concentration (Fig. 7A).
Ten clones were cultured from the second sort of each condition (1 nM MART-1-HLA-A2, with or without serum), and the induced cells were analyzed further for binding to MART-1-HLA-A2. Sequences were obtained from 19 clones, and all but one contained the mutation F45BY. Sorting in the presence of serum yielded three unique mutants: F45BY (7 of 9), F45BY/M97αr (1 of 9), and F91BY (1 of 9). Sorting in the absence of serum yielded six unique mutants: F45BY (4 of 10), F45BY/K44BE (1 of 10), F45BY/E102B8 (1 of 10), F45BY/D100αS (1 of 10), F45BY/D100αC (1 of 10), F45BY/E102B8/K97αE (1 of 10), and F45BY/M97αQ/K41αA (1 of 10). Interestingly, the deep mutational scan of monomer-sorted T1-SCL had demonstrated 4-fold or higher enrichment for many of these substitutions. The F45BY and M97αQ substitutions were enriched 48- and 32-fold, respectively, compared with wildtype residues (Fig. 3), consistent with their representation in the selected clones.

Because we were interested in obtaining interface mutants that improved the Vα/Vβ interaction, clones that contained mutations in the spatially close Phe<sup>159</sup>/Met<sup>97α</sup> pair were chosen for further analysis (F45BY/M97αR and F45BY/M97αQ/K41αA). In addition, the single-site F45BY mutant and single-site F91BY mutant were analyzed further to assess their behavior as single mutations. Further analysis of these mutants was also based on staining with 100 pM MART-1-HLA-A2 (data not shown) and enrichment scores of individual mutations from monomeric MART-1-HLA-A2 selection of T1-SCL. The four unique mutants (F45BY, F91BY, F45BY/M97αR, and F45BY/M97αQ/K41αA) were analyzed by flow cytometry-based ligand titrations with monomeric MART-1-HLA-A2. Surprisingly, the mutants F45BY, F45BY/M97αR, and F45BY/M97αQ/K41αA not only exhibited higher yeast surface levels (i.e. due to increased stability), but they had $K_d$ values in the range of 1.0–2.3 nM, ~50–100-fold higher affinity than the parental T1 TCR and mutant F91BY (Fig. 8A and Table 1). This result suggested that the mutation in common among them (F45BY) accounted for the majority of the affinity increase. Similarly, the F45BY mutation also appeared to be responsible for higher surface display levels compared with the T1 TCR (Fig. 8A).

The unexpected magnitude of the affinity increase (that is, ~50-fold higher compared to T1 TCR) with the interface mutation F45BY raised the question of whether the mutants would retain specificity for the MART-1 peptide, or the mutation might have enabled binding to other peptide-HLA-A2 complexes, perhaps by enhanced interactions with the HLA-A2 helices. To test this, we examined binding of the T1 TCR and all four mutants to a high concentration of tetramers generated with a panel of HLA-A2–restricted cancer or virus peptides (Fig. 8B). Although all of the TCRs bound strongly to the MART-1-HLA-A2 tetramers, none of the eight other complexes tested bound to the interface mutants or the T1 TCR. Thus, the increase in affinity did not result in detectable levels of peptide cross-reactivity, even with multivalent reagents that are capable of detection when the monomeric $K_d$ values are in the micromolar range (51).

### Affinity of soluble mutant TCR F45BY

To show that the affinity and stability improvements associated with the interface mutation F45BY would be transferred to the soluble form of the TCR (i.e. not found only in the yeast-display version), we expressed the T1 and F45BY TCRs as inclusion bodies in *E. coli* and purified by nickel chromatography and gel filtration (SDS-polyacrylamide gel; Fig. 9A, inset), as we have done with other single-chain TCRs (22, 23, 52–54). To compare the binding of the soluble proteins, MART-1-HLA-A2 monomeric complexes were titrated on immobilized soluble T1 TCR or F45BY TCR, and the bound HLA-A2 was detected with anti-β2m antibody–HRP conjugates (Fig. 9A). In this assay, the soluble T1 TCR exhibited a $K_d$ value of 76 nM, and the soluble F45BY TCR exhibited a $K_d$ value of 1.3 nM. Thus, the soluble form recapitulated the effect of the mutation on affinity of the yeast-displayed form.

To determine kinetic parameters of their interaction, we performed a kinetic titration experiment using surface plasmon resonance (SPR) (55). Biotinylated MART-1-HLA-A2 was immobilized on a sensor chip, and various concentrations of soluble, monomeric sTCRs (T1 or F45BY) were injected, without regeneration steps between injections. An unrelated peptide-HLA-A2 complex (WT1-HLA-A2) was immobilized on a reference flow cell to control for nonspecific binding. The association and dissociation phases were monitored for 2 and 3 min, respectively. SPR measurements indicated that the F45BY TCR exhibited ~58-fold increase in affinity ($K_d$ of 0.26 nM), compared with the T1 TCR ($K_d$ of 15.2 nM) with faster on-rate and slower off-rate kinetics (Fig. 9, B and C).

### Thermal stability of T1 and F45BY TCRs

As indicated above, the yeast-displayed F45BY TCR exhibited a higher maximal cell-surface level than the T1 TCR (Fig. 8A), a property that has been shown previously to correlate with increased thermal stability (30, 46). To examine the thermal stabilities of the T1 TCR and F45BY TCR, we took two inde-
The resulting temperature denaturation of F45 from the tetramer-only selection did not differ substantially for T1 TCR or interface mutants (F91) compared to the tetramer-only selection. Yeast-displayed TCRs were titrated with monomeric MART-1 HLA-A2, as described in the legend to Fig. 8A. Resulting data were fit to a one-site-specific binding equation (\( Y = B_{\text{max}} \times X / (K_D + X) \)), where X is ligand concentration, and Y is mean fluorescence units. Average \( K_D \) values from several experiments (n = 7, 1, 2, 2, and 5 for T1, F91Y, F45βY/M97αR, F45βY/M97αQ/K41αA, and F45βY, respectively) are reported.

### Table 1

| Yeast-displayed TCR | \( K_D \) (nM) |
|---------------------|-----------------|
| T1                  | 101 ± 48        |
| F91Y                | 122 ± 29        |
| F45βY/M97αR         | 2.3 ± 0.3       |
| F45βY/M97αQ/K41αA   | 1 ± 0.1         |
| F45βY               | 2 ± 0.5         |

We describe here the results of the most thorough deep mutational scan (37–39, 57, 58) yet conducted with a TCR. The analysis included not only CDR residues, as published recently (40, 41), but also a large number of framework residues that are located on the surface of the V domains or at the interface of the Va and the Vβ. As expected, we observed many substitutions in both the CDRs and the interface that were depleted in the pep-MHC selections (i.e. orange in Fig. 3) because binding would be influenced by both affinity and the presence of properly folded and associated Va and Vβ domains. We also observed, as expected based on our previous studies, a smaller frequency of substitutions in CDR residues that yielded improvements in these properties (i.e. blue in Fig. 3). However, to our surprise, we saw a similar or higher (depending on set enrichment cutoff) frequency of substitutions in the interface residues that yielded improvements in these properties (Fig. 4B). Whereas we anticipated these would be exclusively due to stabilization of the Va/Vβ association, and thus higher yeast surface levels, we discovered by conducting follow-up directed evolution selections that one of the highly enriched substitutions (F45βY) was actually acting to increase the affinity by a remarkable 60-fold.
The identification of a conservative substitution (Phe to Tyr, adding only a single hydroxyl group) that is able to act at a distance of up to 20 Å from the TCR/peptide-MHC-binding interface to increase the affinity by 60-fold and the temperature stability by 6 °C raises the obvious question of mechanism of action. In the absence of a structure for the T1 TCR, we resorted to protein modeling using RosettaBackrub (59) to gain insight into this question. Because the Mel5 TCR (PDB code 3HG1) (43) also binds to MART-1/HLA-A2 and uses the same Vα region (Vα2 or TRAV12-2), we used it as a template for modeling the Vα/Vβ interface of the T1 TCR, with the wildtype Phe or the mutated Tyr at Vβ position 45. Overlay of the top-scoring models suggested that the phenyl ring of Tyr45β might be flipped relative to Phe45β, allowing Tyr45β to engage in polar interactions with Leu96α and Tyr35β (Fig. 11). Although this might explain an improved ability of the Vβ to associate with the Vα (and thus improve the temperature stability and yeast surface levels), it does not suggest how this interaction could be propagated to the pep-MHC-binding site consisting of CDRs, allowing the TCR to bind with 60-fold higher affinity yet maintain peptide specificity.

There may be some precedence for similar long-range effects in the Vβ of a TCR, as we have shown that a Vβ domain engineered for high affinity against the toxin TSST-1 showed cooperativity between two residues that were separated by >20 Å (60). The mechanism involved in that system remains speculative, but it was suggested to involve a conformational dynamic process propagated along strand c of the Vβ. Analogous allosteric mechanisms have been suggested in TCR/CD3 and TCR/peptide/MHC interactions (61–63). Of course, beyond TCR-related systems, there are many other proteins where allosteric effects have been observed at significant distances (e.g. see Refs. 64–68). We presume that a similar mechanism is operating with the Tyr45β. Interestingly, the importance of tyrosine residues at protein-protein interfaces has been demonstrated in minimalist combinatorial libraries using only Ser/Tyr residues, which allow high-affinity interaction with the target by providing a larger interaction interface and mediating more molecular contacts (69, 70). It is possible that the Tyr45β in T1 allows a more favorable interaction with Vα due to this effect, but how this drives an increase in affinity for MART-1/HLA-A2, presumably by allosteric means, remains unknown.
Unlike most scFv fragments derived from the VL and VH domains of antibodies, single-chain TCRs consisting of V\(\alpha\)/H9251 and V\(\beta\)/H9252 domains are not sufficiently stable to allow expression on the yeast surface or in soluble form (35, 46). Previously, we used error-prone PCR-based methods to stabilize the V domains of a TCR, allowing them to be displayed in a single-chain format on yeast or expressed in soluble form (22, 23, 31, 71). Among the classes of mutations that allowed higher surface levels were those that resided at the interface of the V\(\alpha\) and V\(\beta\) domains (16, 35) or were distributed elsewhere in the framework region of V domains (22, 30, 31, 34) that endowed higher thermostability. However, these mutations had not been associated with an increase in the affinity of the TCR for the ligand, perhaps because the selecting agent in these stability studies had not.

**Figure 10. Impact of the F45\(\beta\)Y mutation on the lower affinity T1-WT TCR.** A, yeast-displayed T1 TCR, F45\(\beta\)Y mutant, T1-WT TCR, and T1-WT-F45\(\beta\)Y mutant were stained with varying concentrations of tetrameric MART-1-HLA-A2 prepared with streptavidin-PE. Resultant MFU were plotted against ligand concentration to obtain binding curves. B, yeast-displayed T1 TCR, F45\(\beta\)Y mutant, T1-WT TCR, and T1-WT-F45\(\beta\)Y mutant were stained with varying concentrations of monomeric MART-1-HLA-A2 (biotinylated), followed by streptavidin-PE. Resultant MFU were plotted against ligand concentration to obtain binding curves. Binding curves of T1 and F45\(\beta\)Y are shown in the inset.

**Figure 11. Structures of T1 and F45\(\beta\)Y mutant TCR modeled by RosettaBackrub.** A, selected residues at the V\(\alpha\)/V\(\beta\) interface in Mel5 TCR (PDB code 3HG1) were mutated to residues in T1 or F45\(\beta\)Y (Fig. S1). Using the RosettaBackrub server (http://kortemmelab.ucsf.edu/backrub), 3 models were generated. Overlaid models of T1 (V\(\alpha\) (yellow) and V\(\beta\) (cyan)) and F45\(\beta\)Y (V\(\alpha\) (red) and V\(\beta\) (blue)) are shown. MART-1 (green) is shown in complex with HLA-A2 (gray). B, zoomed-in view of A. Polar interactions between Tyr\(^{45}\) and Leu\(^{96}\) and Tyr\(^{45}\) and Tyr\(^{35}\) are shown by black, dashed lines.
been the ligand but was an antibody to the folded Vβ domain. This suggestion is consistent with the deep mutational scan performed here with the anti-Vβ16 antibody as the selecting agent. Whereas there were substitutions that yielded enrichments in substitutions in T1 TCR with anti-Vβ16 (blue in Fig. 5), the F45βY substitution, identified here as being highly enriched with MART-1-HLA-A2, was not one of them. In contrast, all three deep mutational scans using MART-1-HLA-A2 selections had identified the F45βY substitution among those having the most significant enhancement values (Fig. 3 and Figs. S2 and S3). Interestingly, it has been demonstrated that framework mutations in anti-HIV-1 antibodies are required for broad neutralization of HIV-1 strains. Specifically, a framework mutation A61P (in the VH domain) caused a decrease in thermal stability of the anti-HIV-1 antibody, but it resulted in an increased flexibility of the framework, thus promoting neutralization of several strains of HIV-1 (72).

Efforts to engineer higher affinity antibodies and TCRs have typically relied on the use of directed evolution, made possible by generating libraries of mutants in CDRs, the regions involved in antigen binding. The results here show that it is worth considering the generation of libraries at interfaces, which provide opportunities to increase both the stability and affinity of the proteins. In the case of TCRs, these engineered mutants could be valuable as bispecific agents for soluble therapy directed to cancer HLA antigens (21) or in adoptive T-cell strategies (73).

**Experimental procedures**

**Yeast display, reagents, and flow cytometry**

MART-1 (ELAGIGILTV)-HLA-A2–specific T1 TCR was expressed in single-chain format (Vβ16-linker-Vo2) in yeast-display vector pCT302 and engineered for high affinity (23). Site-directed mutation in T1-WT TCR was generated at position 45 in the β-chain using the QuikChange Lightning kit (Agilent Technologies) as described by the manufacturer. For production experiments, HLA-A2 heavy chain was expressed in E. coli as inclusion bodies and refolded in the presence of human β2-microglobulin and UV-cleavable peptide, KILGFTFYV (where J is 3-amino-3-(2-nitro)phenyl-propionic acid) (74). MART-1-HLA-A2 monomers were prepared by UV-mediated peptide exchange. Because HLA-A2 heavy chain was expressed with a C-terminal BirA biotinylation substrate sequence, biotinylated peptide/HLA-A2 complexes could be detected with streptavidin-phycocerythrin (streptavidin-PE) (BD Pharmingen). Tetrameric MART-1-HLA-A2 was prepared by mixing biotinylated monomer with streptavidin-PE in a 4:1 molar ratio. MART-1 and UV-cleavable peptides were synthesized by GenScript Inc.

To assess binding by flow cytometry, yeast cells displaying T1 TCR (or its libraries or mutants) were stained with various concentrations of biotinylated, monomeric MART-1-HLA-A2. After washing, bound biotinylated MART-1-HLA-A2 was detected by staining with streptavidin-PE (1:100). In certain experiments, yeast cells expressing T1 TCR (or its libraries or mutants) were incubated in pooled normal human serum (Innovative Research) supplemented with 10 mM EDTA at 37 °C for 30 min before staining with monomeric MART-1-HLA-2. In some experiments, MART-1-HLA-A2 streptavidin-PE tetramers or anti-Vβ16 monoclonal antibody (clone TAMAYA1.2, Beckman-Coulter) were used to stain yeast cells. The anti-Vβ antibody was used at a 1:100 dilution followed by goat anti-mouse IgG (H + L), F(ab’)2, Alexa Fluor® 647 conjugate (1:100) (Life Technologies, Inc.).

**Construction, selection, and deep sequencing of single-codon libraries in T1 TCR**

SCLs in T1 scTCR were constructed by PCR as described previously (40). Primers containing a single degenerate codon (NNK) at targeted locations were synthesized by Integrated DNA Technologies and used to create PCR products with degeneracy in one codon at a time via splice overlap extension PCR. Overall, 87 such PCR products were prepared and were pooled and transformed into the EBY100 strain of Saccharomyces cerevisiae, along with digested pCT302 vector to allow construction of SCLs by homologous recombination. Resultant libraries in yeast contained $3.4 \times 10^8$ independent transformants, which exceeded by several orders of magnitude the expected diversity (87 positions $\times 32$ codons = 2784). Libraries obtained were analyzed by flow cytometry as described above and sorted with 10 nm MART-1-HLA-A2 monomer or 100 nm tetramer or anti-Vβ16 (1:100) by FACs. An additional sort was performed with 100 nm tetramer, where yeast cells displaying SCLs were incubated at 45 °C for 30 min before treatment with tetramer. The top 1% of cells with bound ligand were collected from each sorted population and expanded at 30 °C. Equal numbers of cells from sorted and unsorted libraries were lysed, and DNA was isolated (Zymo Research Corp.). Genomic DNA was removed by treatment with exonuclease I and A-exonuclease (New England Biolabs, Inc.), and plasmid DNA was purified. Resulting DNA was subjected to deep sequencing, as described previously (39). Vα and Vβ fragments were prepared by PCR for deep sequencing by adding sequences that allow annealing to Illumina MiSeq sequencing primers, followed by adding sequences that allow annealing to flow cell (“adapters”), and allow unique identification of DNA isolated from unsorted or sorted libraries (“indexes”). Amplified PCR products were purified by gel extraction and sequenced using 250-nucleotide paired-end sequencing runs on a MiSeq sequencer. Resulting sequences were analyzed by Enrich software (75, 76) to obtain enrichment ratios for each substitution at library locations compared with the wildtype residue (enrichment ratio = frequency of the substitution in sorted library/frequency of the substitution in unselected library), and sequence fitness landscapes for each sorting condition were created.

**Construction and selection of interface libraries in T1 TCR**

Like SCL constructed in various regions of T1 TCR during deep mutational scan, additional libraries were constructed at possible interface residues by PCR-based methods as described above. Choice of residues to make interface libraries was guided by deep mutational scans of T1 and structural analysis of existing Vα2-containing TCRs (40, 43–45), searching for possible binding partners of each interface residue in a 4-A space. Based on deep mutational scans and structural analysis, SCL in 5 Vα residues (Phe52a, Lys41α, Ser42α, Met47a, and Asp400a) and 11
T-cell receptor interface residues

Vβ residues (Met34β, Lys41β, Glu42β, Lys44β, Phe45β, Met60β, Phe91β, Val101β, Glu102β, and Gly106β) were used. Additionally, PCLs were constructed in seven Vα/Vβ pairs (Lys41β/Asp100x, Glu42β/Asp100x, Lys44β/ Met97x, Phe45β/Met97x, Phe91β/Lys41x, Phe91β/ Ser42x, and Gly106β/Ser42x). PCR products for SCL were pooled separately from PCL and transformed into yeast. Resultant libraries contained 2 × 10⁷ and 5 × 10⁷ independent transformants for pooled SCL and PCL, respectively, which exceeded by several orders of magnitude the expected diversity (16 positions × 32 codons = 512 for SCL; 7 × (32 × 32 codons) = 7168 for PCL). Equal numbers of yeast cells displaying SCL or PCL were mixed and sorted by FACS twice with 1 nM MART-1-HLA-A2 (with or without prior exposure to pooled normal human serum (supplemented with 10 μM EDTA) at 37 °C for 30 min). The top 1% of cells bound to MART-1-HLA-A2 were collected, and individual mutants from each sorting condition were isolated that were analyzed by sequencing or flow cytometry as described above.

**Binding of soluble T1 and F45β TCRs to MART-1-HLA-A2 by ELISA**

T1 scTCR and the F45β mutant scTCR were cloned in pET28a for expression in E. coli as inclusion bodies that were refolded as described previously (23). Soluble, monomeric protein was coated on wells of an ELISA plate (5 μg/ml), followed by incubation with various concentrations of MART-1-HLA-A2. Bound MART-1-HLA-A2 was detected with anti-β2m microglobulin antibody conjugated to horseradish peroxidase (Bio-Rad). The addition of 3,3',5,5'-tetramethylbenzidine substrate (KPL, Inc.) allowed development of colored product that was detected by measuring absorbance at 450 nm.

**Measurement of kinetic parameters of binding of soluble T1 and F45β TCRs to MART-1-HLA-A2 by surface plasmon resonance**

Biotinylated MART-1-HLA-A2 or WT1-HLA-A2 was coated on sensor chip CAP (GE Healthcare) mounted on a Biacore 3000 instrument set at 25 °C. Soluble, monomeric scTCRs (T1 or F45β) were injected over the coated surface at a flow rate of 10 μl/min. All proteins were buffer-exchanged to SPR running buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20). Resulting data were analyzed using Biarevaluation version 4.1 software (55).

**Thermostability assays of T1 and F45β TCRs**

Thermostability of T1 and F45β TCRs were measured in yeast-displayed form as well as in soluble form. To measure thermostability in yeast-displayed form, yeast cells displaying T1 or F45β TCR were incubated at temperatures ranging from 30 to 80 °C for 30 min and then stained with monomeric, biotinylated MART-1-HLA-A2 followed by staining with streptavidin-PE. The percentage decrease in fluorescence relative to fluorescence at 30 °C was calculated for each mutant and plotted against temperature to obtain melting curves and Tm (46). To measure thermostability in soluble form, solutions containing soluble monomers of T1 or F45β TCRs (5 μM), 5 × SyproOrange, and 1 × assay buffer were aliquoted into wells of a 384-well plate and exposed to increasing temperature (at a rate of ~1 °C/min) in a Taqman ABI 7900 real-time PCR instrument. Resulting fluorescence obtained was plotted against temperature using OriginPro software. First derivatives of bi-Gaussian function fitted data were used to precisely calculate Tm.

**Protein modeling by RosettaBackrub**

For protein modeling, we used Mel5 TCR (PDB code 3HG1) (43) as a template and RosettaBackrub (59) (http://kortemmelab.ucsf.edu/backrub) as the modeling tool. Mel5 TCR (like T1) also binds to MART-1-HLA-A2 and uses the same Vα region (TRAV12–2) as T1. For modeling the Vα/Vβ interface of T1 or F45β TCRs, we mutated a stretch of amino acids in Mel5 (92VAGKST97 in Vα and 43LQLLYF39 in Vβ) to residues in T1 or F45β TCR (92SSDFLM97 in Vα and 43IK(FY)LLHF49 in Vβ) (Fig. S1). The top scoring models of each were analyzed and overlaid in PyMOL (Schrödinger, LLC).

**Author contributions**—D. M. K. and P. S. designed the study. P. S. performed experiments. D. M. K. and P. S. analyzed the data and wrote the manuscript. D. M. K. and P. S. reviewed the results and approved the final version of the manuscript.

**Acknowledgments**—We thank Monika Kizerwetter and Dylan Blaha for experimental assistance and Daniel Harris and Erik Procko for advice with the deep mutational scans. We also thank members of the Flow Cytometry, Core DNA Sequencing, High-Throughput Sequencing, and Protein Sciences facilities of the Roy J. Carver Biotechnology Center at the University of Illinois Urbana Champaign for technical assistance.

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