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Combinations of Affinity-Enhancing Mutations in a T Cell Receptor Reveal Highly Nonadditive Effects within and between CDRs and Chains

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Abstract

Understanding the energetic and structural response to multiple mutations in a protein-protein interface is a key aspect of rational protein design. Here we investigate the cooperativity of combinations of point mutations of a T cell receptor (TCR) that binds *in vivo* to the HLA-A2 MHC and a viral peptide. The mutations were obtained from two sources: a structure-based design study on the TCR α chain (9 mutations) and an *in vitro* selection study on the TCR β chain (4 mutations). In addition to combining the highest affinity variants from each chain, we tested other combinations of mutations within and among the chains, for a total of 23 TCR mutants that we measured for binding kinetics to the peptide/MHC. A wide range of binding affinities was observed, from 2-fold to 1000-fold binding improvement over wild-type, with significant nonadditive effects observed within and between TCR chains. This included an amino acid dependent cooperative interaction between CDR1 and CDR3 residues that are separated by over 9 Å in the wild-type complex. When analyzing the kinetics of the mutations, we found that the association rates were primarily responsible for the cooperativity, while the dissociation rates were responsible for the anticooperativity (less-than-additive energetics). Based on structural modeling of anticooperative mutants we determined that side chain clash between proximal mutants likely led to nonadditive binding energies. These results highlight the complex nature of TCR association and binding and will be informative in future design efforts that combine multiple mutant residues.

Protein-protein interactions are responsible for a large variety of biological processes, including humoral and cellular immune responses, viral capsid assembly, and inhibition of enzyme function. While much progress has been made to understand protein-protein interactions on a molecular and structural level, due to their enormous complexity there is still much to be known before the dynamic interplay between residues in an interface is understood and can be modeled reliably.

One important area of protein-protein interaction research is the energetic and structural impact of multiple simultaneous mutations in a protein interface. Initial research in this area

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SUPPORTING INFORMATION AVAILABLE Figure with $\Delta\Delta G_{\text{on}}$ and $\Delta\Delta G_{\text{off}}$ for all measured A6 TCR mutants. This material is available free of charge via the Internet at <http://pubs.acs.org>.

determined that the majority of combinations of mutations have additive energetics, however nonadditivity is seen in cases where the interface is not rigid or the mutant residues are in close proximity to one another (1). Based on binding data from mutagenesis studies and protein structural data, some have found that protein interfaces are organized into a modular framework, with each module composed of a cluster of interacting residues that have cooperative binding behavior (where the measured binding energy improvement, $\Delta\Delta G$, is larger than the sum of individual $\Delta\Delta G$ s), whereas separate modules act independently and have additive binding energetics (2-4). However, a recent study of combinations of mutations on a T cell receptor (TCR) obtained from phage display in fact found significant cooperativity within *and between* modules (also referred to as hot regions) (5). The inter-hotspot cooperativity was exhibited between clusters separated by over 20 Å; the authors attributed this to TCR flexibility and propagation of the energetics through a “dynamic structural network”. Combinations of sets of mutations from multiple experiments have not been studied extensively, though one study found that when mutant modules of human growth hormone (hGH) from separate phage display experiments were combined, they behaved in a largely additive fashion (although there was cooperativity *within* the modules) (6). Rational design protocols have been utilized to improve the affinities of antibodies for antigens by combining point mutations, with the highest affinity antibody mutants displaying additivity (7) and cooperativity (8) between point mutations.

In this study, we investigated the cooperative nature of protein binding by measuring the binding kinetics of combinations of point mutants in a TCR/peptide/MHC interface. This system is of critical interest in immunology and vaccine design (9,10), as well as cell and antigen targeting (11). Several studies have featured improving the affinity of various TCRs for peptide/MHC complexes using *in vitro* selection methods, as discussed in several reviews (12-14). We produced mutations on the A6 TCR, which is a human $\alpha\beta$ T cell receptor protein that binds *in vivo* to the HLA-A2 MHC and a 9-mer peptide from the Tax protein of human T-lymphotropic virus 1 (HTLV-1). The structure of this complex has been determined by X-ray crystallography (15), and its binding kinetics and thermodynamics have been reported in several studies (16-19).

The mutations we selected for combination are from two sources: a phage display study that improved binding approximately 700 times over wild-type (20), and a structure-based design study we performed that improved binding approximately 100 times over wild type (19). As the structure-based design mutants are located on the α chain and the phage display mutants are located on the β chain, we tested the combination of the two high-affinity mutant chains, as well as other combinations of mutations within and among the chains, leading to a total of 23 combinations of mutations that we produced and measured for peptide/MHC binding. While many of the combinations exhibited cooperativity and additivity, three had a significant degree of anticooperativity (also referred to as negative cooperativity), with measured energies less than expected from additivity of the component point mutations. We determined the contributions of specific residues to the non-additive nature of the cooperative (and anticooperative) mutants, as well as the contributions of the association and dissociation rate. To further explore these experimental results, we performed an *in silico* analysis of the anticooperative mutations to determine their impact on the structure of the complex, and found side chain clash between affinity-improving point mutations leading to anticooperativity.

MATERIALS AND METHODS

Energy Change Calculation

$\Delta\Delta G_{\text{on}}$ and $\Delta\Delta G_{\text{off}}$ were computed using the association rate (k_{on}) and dissociation rate (k_{off}) as follows:

$$\Delta\Delta G_{\text{on}} = RT \times \ln(k_{\text{on, wild-type}}/k_{\text{on, mutant}}) \quad 1)$$

$$\Delta\Delta G_{\text{off}} = RT \times \ln(k_{\text{off, mutant}}/k_{\text{off, wild-type}}) \quad 2)$$

In these equations, R is the gas constant, and T is the temperature in Kelvin. The total binding energy change, $\Delta\Delta G$, is the sum of these terms:

$$\begin{aligned} \Delta\Delta G &= RT \times \ln(K_{\text{D, mutant}}/K_{\text{D, wt}}) = RT \times \ln(k_{\text{off, mutant}}k_{\text{on, wild-type}}/k_{\text{on, mutant}}k_{\text{off, wild-type}}) \\ &= \Delta\Delta G_{\text{on}} + \Delta\Delta G_{\text{off}} \end{aligned} \quad 3)$$

Where K_{D} is the equilibrium dissociation constant, equal to $k_{\text{off}}/k_{\text{on}}$.

Protein Expression and Purification

Proteins were expressed and purified as described previously by Haidar et al. (19). Briefly, mutations were introduced to the TCR constructs via site-directed mutagenesis using standard PCR protocols. HLA-A2, β 2M, wild-type and mutant TCR α and TCR β chains were expressed separately as inclusion bodies in *E. coli*. The inclusion bodies were refolded using protocols based on those of Garboczi et al. for the TCR α and TCR β (21), and for refolding the Tax peptide (LLFGYPVYV) with HLA-A2 and β 2M to form the MHC/peptide complex (22). Fast protein liquid chromatography (FPLC) using a size exclusion column (rather than dialysis and ion exchange) was used to separate the folded proteins from aggregates, resulting in faster purification of the proteins than the previously published method (21).

Measurement of Binding Kinetics

Binding of wild-type and mutant TCRs to the Tax/HLA-A2 MHC complex was measured using a Biacore 3000 surface plasmon resonance (SPR) biosensor at 25°C. HBS-EP (0.01 M HEPES (pH 7.4), 0.15 mM NaCl and 3 mM EDTA, 0.005% v/v Surfactant P20) was used as a running buffer during binding affinity measurements. Approximately 400 Response Units (RU) of TCR were immobilized onto a CM5 chip using the standard amine coupling procedure. While Tax/HLA-A2 complex was injected over immobilized TCR at a flow rate of 100 Δ l/min, followed by dissociation using running buffer. Injection times were either 90 seconds or 150 seconds, with the latter time used to allow for adequate association of the high affinity complexes. Tax/HLA-A2 concentrations were gradients of two-fold dilutions, with at least three concentrations per gradient, and three gradients per experiment.

After dissociation, regeneration of TCR surface was achieved with a single two minute injection of 0.01 M HEPES (pH 7.4) 1 M NaCl (for lower affinity complexes, $K_{\text{D}} > 10^{-8}$), or two 10 second injections of 10 mM HCl (for high affinity complexes, $K_{\text{D}} < 10^{-8}$). The latter regeneration condition was selected after testing the β chain MSAQ mutant from Li et al. (20) using the HEPES/NaCl solution; only one measurable concentration was obtained for kinetic analysis due to incomplete TCR surface regeneration. Later measurements of high affinity mutants using the 10 mM HCl regeneration buffer yielded effective surface regeneration and gradients that could be globally fit.

BIAevaluation software version 4.1 (Biacore Inc.) was used to analyze the results from the kinetic experiments. After double-referencing to remove artifacts from nonspecific binding (23), simultaneous global fitting of the data for each concentration gradient to a 1:1

Langmuir model was performed to determine the association rate (k_{on}), dissociation rate (k_{off}), and dissociation constant ($K_D = k_{off}/k_{on}$). Other models, such as the two-step binding model, did not improve fit quality substantially over the 1:1 Langmuir model, which yielded high quality fits to binding data for all mutants. For each mutant tested, at least three different Tax/HLA-A2 concentration gradients were used to compute the kinetic parameters and the sample standard deviation.

Simulation and Evaluation of Mutant Protein Structures

Mutant structures were simulated using the protein modeling program Rosetta (24), starting with the crystal structure of the wild-type A6 TCR/Tax peptide/HLA-A2 MHC complex (15) which was downloaded from the Protein Data Bank (25), accession code 1AO7. Structural modeling with Rosetta was performed using the interface mutagenesis module (24), keeping the protein backbones fixed, and packing the mutant side chains using a rotamer-based search. Standard rotamers were augmented with extra chi1, chi2, and chi3 rotamers for all residues with at least 12 neighboring residues within a 10 Å cutoff (specified by the “-ex1 -ex2 -ex3 -extrachi_cutoff 12” flags to Rosetta on the command line).

Structural models were then scored using ZAFFI, a function optimized to evaluate the binding energy changes of mutant complexes (19). This function uses a weighted scoring function with terms for van der Waals, Lazaridis-Karplus solvation (26), and the ACE statistical potential (27). For each mutant structure scored by ZAFFI, the score of the wild-type complex was subtracted away to produce the predicted energetic change of the mutation.

Figures

Figures representing protein structures were generated using PyMOL (www.pymol.org), and graphs were generated using gnuplot (www.gnuplot.info).

RESULTS

Positions of TCR Mutations

To evaluate binding cooperativity in the A6 T cell receptor, we produced a set of 23 mutant TCRs with combinations of point mutations that had been measured individually for binding kinetics to Tax-HLA-A2. The mutations in the TCR α chain are at positions 26, 27, 28, 51, and 100, located in complementarity determining regions (CDRs) 1 (residues 26-28), 2 (residue 51), and 3 (residue 100), and have wild-type residue identities D, R, G, S, and S respectively. The four mutated positions on the TCR β chain are 99-102 (located in CDR3), for which the wild-type sequence is AGGR. The mutations on the TCR α chain were originally identified and tested in a structure-based design study (19), while the four mutations on the TCR β chain were produced together in a high affinity mutant in a phage display study (20). All of these point mutations were measured individually for kinetics by Haidar et al. and showed improved affinity, with the exception of α S100A, which led to negligible increase in affinity, and β R102Q, which led to an approximately 2-fold decrease in affinity (19).

Figure 1 shows the location of the mutations on the structure of wild-type TCR interface. While the α S100 and β A99-R102 residues are close to one another near the center of the interface (with just 5.3 Å separating α S100 and β G100), residues α D26-G28 and α S51 are located more on the periphery of the interface, approximately 9.5 Å and 14.4 Å from the α S100 residue, respectively, and 11.4 Å from each other. Among residues α D26-G28, α G28 is the closest to the center of the interface and the mutated residues in the other CDRs.

The TCR combination mutants were expressed, folded, and tested for binding kinetics to Tax/HLA-A2 using surface plasmon resonance, as described in the Materials and Methods section. Table 1 gives measured binding kinetics of the mutants, and representative binding sensorgrams for wild-type A6 TCR and five mutants are shown in Figure 2. For simplicity, mutant TCRs with multiple α chain mutations are denoted by the amino acid identities at positions 26, 27, 28, 51 and 100, and TCRs with multiple β chain mutations are denoted by the amino acid identities of residues 99, 100, 101, 102, with mutant residues in bold.

We also computed the changes in activation energies of association and dissociation, $\Delta\Delta G_{\text{on}}$ and $\Delta\Delta G_{\text{off}}$, defined in Equations 1 and 2. Such formulations have been used in previous studies to express changes in kinetic rates in energetic terms (28,29). $\Delta\Delta G_{\text{on}}$ is directly related to the ϕ value ($\phi = \Delta\Delta G_{\text{on}} / \Delta\Delta G$), which has been used to analyze the contribution of inter-residue interactions to TCR association (30,31). Supporting Figure 1 gives the $\Delta\Delta G_{\text{on}}$ and $\Delta\Delta G_{\text{off}}$ for the measured TCR combination mutations, as well as the individual point mutations measured previously (19).

Measured Binding Kinetics

We measured binding kinetics for 14 combination mutations that contained mutations in the α chain alone, shown in Table 1. For the wild-type and the combination mutants **WFGMT** and **WFTMT**, the kinetic rates were reported previously (19). Certain other mutants of the α chain were also produced for testing (namely **WFIMT**, **WFMMT**, **WFLMT**, **WRIMT**, **WRLMT**), but binding was unmeasurable due to poor folding, likely because of the hydrophobicity of the mutant residues. Of the measured mutants, many had association rates similar to or slightly lower than the wild-type association rate of $5.1 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, ranging from approximately 5-fold slower (**WRTMT**) to nearly 3-fold faster (**DRMST**). Notably, the three mutants with the highest measured association rates contained the α G28M mutation, which had the highest association rate of all individual point mutations reported previously (19). The dissociation rates varied more than the association rates, with the largest change seen for **WFGMT**, which had a dissociation rate (and total K_D) approximately 100 times lower than that of the A6 wild-type TCR. The large contribution of the dissociation rate to the total energy change (relative to the association rate) was seen in nearly all measured A6 TCR mutants (Figure S1).

Even greater improvements in binding affinity were seen for the mutants in the TCR β chain, which were all located in the CDR3 loop. The most improvement was seen for the quadruple mutant **MSAQ**, identified through phage display in a previous study (20). Our affinity measurements for this mutant, although lacking replicate measurements, are commensurate with the values presented in that work (our measured K_D was 2.8 nM, compared with 2.5 nM reported by Li et al.). For this mutant, a modest decrease in association rate was offset by a drastic improvement in dissociation rate over the wild-type, leading to nearly 800-fold improvement in binding affinity. The triple mutant **MSAR**, which does not contain the β R102Q substitution, has approximately 2-fold lower binding affinity than **MSAQ**, due to reduced association and faster dissociation. Still lower binding was observed for double mutants **MSGR** and **AGAQ**, which comprise the first two and last two point mutations in the **MSAQ** mutant, respectively, each with only approximately 10-fold binding improvement over the wild-type TCR.

We combined the β chain mutations with the α chain mutations from structure-based design and measured their binding kinetics, which are shown in the third portion of Table 1. These featured strong improvements (i.e., decreases) in the dissociation rates, and generally slower association rates compared with the wild type. The combination of point mutation α D26W with the phage display mutant **MSAQ** led to improved binding, with a more than 4-fold improved dissociation rate over **MSAQ**, and an association rate 2-fold less than that of

MSAQ alone. The half-life of binding ($t_{1/2}$, equal to $\ln 2/k_{\text{off}}$) is 4.3 hours for the D26W-**MSAQ** mutant, versus 6.3 seconds for the wild-type TCR. The SPR sensorgram for this mutant, shown in Figure 2, indicates the slow dissociation rate.

We also tested larger combinations of mutations, including the combination of α chain mutation **WFGMT** and β chain mutant **MSAQ**, which were the highest affinity mutants from each individual chain. Surprisingly, for that combination we observed a binding affinity (K_D) of 6.2 nM, which is greater than **WFGMT** alone (21 nM) but less than **MSAQ** alone (2.8 nM). To further probe the combinations of mutant chains we tested the **WFGMS-MSAQ** mutant; in this case a reversion of the α S100T in **WFGMT** to wild-type serine was tested to explore the possible linking of that position to the **MSAQ** mutation in the β chain due to their spatial proximity (as is shown in Figure 1). The binding affinity of **WFGMS-MSAQ** is improved slightly over **WFGMT-MSAQ**, primarily via association rate improvement, indicating that the mutant α S100T was possibly hindering the association process when combined with **MSAQ**.

We noted lower response levels in Biacore from various combinations of mutant α and β chains (including G28T-**MSAR** and D26W-**MSAQ** in Figure 2); we believe this is due to reduced solubility of these TCR mutants and thus lower levels of active protein on the chip surface. To ensure specificity of combined α and β chain mutants, we tested the **WFGMT-MSAQ** mutant TCR for binding to three HLA-A2 bound HIV peptides (sequences: SLYNTVATL, ILKEPVHGV, VIYQYMDDL), and no measurable binding was detected using Biacore (sensorgrams not shown). The specificities of the high affinity mutants from each chain, **WFGMT** and **MSAQ**, have been confirmed in previous studies (19,32).

Cooperativity of TCR Mutations

To explore the cooperative nature of the combinations of mutations presented in Table 1, we compared their measured kinetics with what would have been predicted with additivity from the individual point mutations. Figure 3 provides the cooperative $\Delta\Delta G$, the difference between the measured $\Delta\Delta G$ and the sum of $\Delta\Delta G$ from component point mutations, for all measured combinations of TCR mutations. Table 2 gives the cooperativity in terms of total binding energy ($\Delta\Delta G$) as well as $\Delta\Delta G_{\text{on}}$ and ΔG_{off} . To further illustrate the nonadditive and kinetic trends observed among the mutations, Figure 4 contains scatter plots of additive versus measured $\Delta\Delta G$ values for all combinations of TCR mutants. Figure 4 indicates that, for the tested TCR mutants, the cooperativity is largely seen in the association rate (Figure 4B), whereas the dissociation rate is responsible for the anticooperativity (Figure 4C).

α Chain Cooperativity

While many mutants were additive, there are several instances of cooperativity and anticooperativity among the combination mutations in the α chain. Using a cooperativity cutoff of $\Delta\Delta G < -0.5$ kcal/mol (also employed by Moza et al. (5)), there is one cooperative mutant that contains only two mutant residues: **DRMST**. It has the point mutations α G28M and α S100T, and a cooperativity of -0.77 kcal/mol, primarily due to the association rate. As noted in Figure 1, in the structure of the wild-type TCR in complex with Tax/HLA-A2 these residues are not adjacent and are separated by over 9 Å. All other measured mutants at those two positions, including I, L, and T for α G28 (**DRIST**, **DRLST**, and **DRTST**) and A for α S100 (**DRISA**, **DRLSA**, **DRTSA**, **DRMSA**), did not show any significant cooperativity. The degree of cooperativity seen in **DRMST** persists with the addition of α S51M (**DRMMT**), suggesting that the α S51M mutation is additive in the context of the other two mutated sites. Given its distance from the other residues and location on the periphery of the interface (Figure 1), it is not surprising that α S51M behaves additively. α S51M also behaves additively when combined with the additive double mutant **DRTST** to produce the triple

mutant **DRTMT**. When the mutant α R27F is combined with the triple mutant **DRMMT** (to generate **DFMMT**), the degree of cooperativity decreases from -0.75 to -0.19 kcal/mol and leads to a lower binding affinity, likewise addition of α R27F to **DRTMT** results in slight anticooperativity in the mutant **DFTMT** (leaving the binding affinity essentially the same as **DRTMT**). Given its proximity to α G28, it is possible that the nonadditive behavior of α R27F with the triple mutations is due to altered backbone dynamics or conformation of the CDR1 α .

We explored other combinations of α chain mutations that included the point mutant α D26W, resulting in both cooperative and anticooperative energetics. The strongest binding α chain mutant, **WFGMT**, exhibited a cooperative $\Delta\Delta G$ of -0.56 kcal/mol due to the association rate, leading to a -2.59 kcal/mol total $\Delta\Delta G$. Given the additivity of α S51M in the context of other mutations and its distance from the CDR1 residues (13 Å from α D26 and 11.5 Å from α R27 in the wild-type structure), we reason that interactions between α D26W and the other mutant residues, α R27F or α S100T are responsible for the cooperativity. The other combination α chain mutations including α D26W, **WRMMT**, **WRTMT**, and **WFTMT**, showed strong anticooperativity, greater than 1.3 kcal/mol, due to the dissociation rate (these mutants are outliers in Figure 4A and 4C). As other mutants with α D26W, α S51M, and α S100T (**WFGMT**) and α G28T or α G28M, α S51M and α S100T (**DRMMT**, **DRTMT**) do not possess anticooperative energetics, and α R27F does not increase the anticooperativity when added to **WRTMT** (in the mutant **WFTMT**), combinations of the mutation α D26W along with either α G28M or α G28T seem to be responsible for the anticooperativity. As these residues are spatially proximal in the CDR1, it is possible that some steric hindrance is responsible for the anticooperativity; the structural implications of these mutant combinations will be discussed in more detail later.

β Chain Cooperativity

In contrast to the anticooperativity and additivity observed among the α chain mutants, the four mutants in the TCR β chain were all cooperative when combined. The double mutants **AGAQ** and **MSGR** both showed some cooperativity between the adjacent point mutations (the latter had a cooperativity of -0.41 kcal/mol which was slightly less than the threshold of -0.5 kcal/mol). When adding the β G101A mutant to **MSGR** to produce **MSAR**, the cooperativity (and consequently the binding affinity) improved dramatically to -1.82 kcal/mol. The cooperativity improved further for the **MSAQ** mutant (which included β R102Q), to -2.66 kcal/mol, yielding a $\Delta\Delta G$ of -3.93 kcal/mol. While both the association and dissociation rates were responsible for the cooperativity of **MSAQ**, the association rate had a larger contribution (-1.60 kcal/mol). It is worth noting that the affinity improvement from **MSAR** to **MSAQ** is approximately equal to improvement from β G101A to **AGAQ**, suggesting that the adjacent mutation β G101A is responsible for the cooperativity observed when adding β R102Q to **MSAR**. Even the sum of $\Delta\Delta G$ from the two pairs of mutations (**AGAQ** and **MSGR**) comprising the **MSAQ** mutant (-3.48 kcal/mol) is somewhat less than the measured $\Delta\Delta G$ of **MSAQ** (-3.93 kcal/mol), indicating cooperativity among (in addition to within) these pairs of positions.

α and β Chain Cooperativity

Combinations of the mutant α and β chains were primarily cooperative when compared with the sum of the component point mutations. Strikingly, the association rate cooperativity of approximately -1.60 kcal/mol persists for all combinations of α chains with **MSAQ** (including wild-type α chain), despite widely varying dissociation rate additivity. This consistency can be seen in the linear trend of these mutants in Figure 4B, and suggests that **MSAQ** is responsible for the association rate shift and dominates the association kinetics in the context of mutated α chains.

When we compared the measured kinetics with the kinetics obtained by adding the contributions from each mutant chain (inter-chain cooperativity; Table 3), however, there is considerably less cooperativity, i.e., the measured energetic improvement is not as large as would be expected from additivity between the individually measured mutant chains. This is most apparent for **WFGMT-MSAQ**, which would have $\Delta\Delta G$ of -6.65 kcal/mol from additivity between chains but instead has a measured $\Delta\Delta G$ of -3.46 kcal/mol, which represents over 200-fold difference in binding affinity (K_D). The smaller and more distant mutants on the α chain became slightly more additive with the β chain mutants (though still anticooperative), with **D26W-MSAQ**, **G28T-MSAQ**, and **G28T-MSAR** having inter-chain cooperativities of 0.97, 0.73, and 0.43 kcal/mol respectively. The inter-chain anticooperativity in Table 3 is primarily due to the dissociation rate, as with the anticooperative mutants from the α chain alone (**WRMMT**, **WRTMT**, and **WFTMT**), suggesting a context-dependent alteration of the bound state for one or both mutant chains.

Structural Modeling of Anticooperative Mutations

Previously we utilized Rosetta (24) to generate structural models for individual TCR point mutations bound to peptide/MHC, leading to considerable accuracy for predicted $\Delta\Delta G$ values for this system (19). After noting the anticooperativity for mutants **WRMMT**, **WRTMT**, and **WFTMT**, we investigated the structural models of these mutations.

Models for the TCR mutants **WRMMT** and **WRTMT** are shown in Figure 5. Although the side chain positions of α D26W, α G28M and α G28T are identical to those in the models of the individual point mutations, surprisingly there is some side chain clash seen in the models for their combination, with less than 3 Å between non-hydrogen atoms of the two side chains. When utilizing the scoring function ZAFFI (19) to evaluate the inter-residue energetics, there is a van der Waals energetic penalty of approximately 0.95 kcal/mol due to clash between α D26W and α G28T, and 0.62 kcal/mol due to clash between α D26W and α G28M. This clash was ignored by the ZAFFI function initially, because intra-chain clash was normally minimal and led to scoring function noise. Such clash is the likely reason behind the anticooperativity of the mutations **WRMMT**, **WRTMT**, and **WFTMT**. The mutant α R27F (in **WFTMT**, not shown in figure), which does not seem to affect the anticooperativity, is directed away from the TCR side chains at positions 26 and 28 on the α chain in the Rosetta models, and thus is not predicted to interact with them. We also searched for similar clashes in models of the other measured A6 combination mutations and none were identified.

DISCUSSION

In this study we measured binding for combinations of mutations of a TCR that has been engineered using two methods, allowing us to characterize cooperativity within and between sets of mutations in the same binding interface. As with the results for individually engineered A6 TCR chains reported previously (19,20), the greatest improvement was seen for the dissociation rates, while the association rate did not vary substantially among TCR mutants. Many designed TCRs also have greater improvements in dissociation rate (aside from the A6 TCR, 7 out of 9 engineered TCRs had greater dissociation rate versus association rate improvements in a recent review (33)), suggesting that this route of binding improvement is not unique to the A6 TCR. Sub-optimal dissociation likely results from the fact that TCRs with long binding half-lives are removed *in vivo* during negative selection in the thymus. Additionally, dissociation rate improvements in the case of the designed A6 point mutants resulted in part from the design algorithm, which stabilized the bound state and featured shape complementarity and desolvation scoring terms (19).

In analyzing the kinetic values from SPR, we used the simple 1:1 Langmuir binding model to obtain values of association and dissociation kinetics (and the dissociation equilibrium constant, K_D), which, given the complex nature of TCR/pepMHC binding, is certain to overlook molecular events taking place during the interaction process. As noted by others (34,35), protein protein binding often occurs with a “transition state” intermediate between unbound and bound proteins, shown as AB^* in Equation 4 for the binding of proteins A and B:

In the above equation, the first step is determined by diffusive and long range electrostatic forces, while the change from intermediate to bound state is guided by short-range interactions and minor structural adjustments to form the bound protein complex. By using a Langmuir 1:1 binding model, intermediate states are not dealt with explicitly (i.e. individual terms k_1 , etc. dealing with the encounter complex are not given), and just the overall kinetic values for association and dissociation are determined. In a comprehensive mutagenesis study of the TEM-1/BLIP interaction, the authors discussed that though in reality the transition state is likely formed, if it is in steady-state and does not accumulate over time it does not need to be considered in calculating the K_D , and the 1:1 Langmuir model can be used (34). In the same study, it was found that SPR using the 1:1 model for analysis was accurate in determining relative $\Delta\Delta G$ values between wild-type and mutant (while less accurate for absolute binding energetic values), when comparing SPR to other measurement systems. In the present study, as with our previous report of A6 TCR point mutations (19), all TCR mutants measured fit well to a 1:1 Langmuir binding model, which has been used by a number of other laboratories to analyze TCR interaction with pepMHC (17,30,36,37), despite strong evidence of conformational change upon binding (38,39). Others have noted the difficulty of detecting transition state complexes using SPR for TCR recognition (40) and protein complexes in general (41), due to the temporal resolution of such techniques, leading some to utilize less direct methods such as ϕ value analysis (30,31) and analysis of kinetic cooperativity (42) to infer the structure and dynamics of the binding transition state.

In this study, the measured combinations of A6 TCR mutations yielded several examples of nonadditive energetics. Within the α chain there was a notable example of cooperativity, between mutations $\alpha G28M$ and $\alpha S100T$, due to the association rate. Nonadditivity in the association rate is said to be due to interaction between residues in the binding transition state (41); this has been applied in analysis of mutants of the barnase-barstar complex (43). In the structure of the wild-type A6 TCR in complex with Tax/MHC, these residues are not particularly close (they are separated by 9.5 Å based on all atoms, with the $C\alpha$ atoms separated by over 13 Å), raising the question of whether these residues are close enough to permit interaction prior to binding. Unfortunately the structure of the unbound A6 TCR has not been crystallized, precluding an analysis of the unbound state for this system, but based on data for other systems, TCRs are known to exhibit plasticity in their CDR loops upon binding to peptide/MHC (44). A review of 12 sets of unbound and bound TCR structures revealed that the CDR3 α , CDR3 β and CDR1 α loops have the highest degree of average movement upon peptide/MHC binding, with average RMSDs (root mean square distances) of 4.7 Å, 3.8 Å and 2.8 Å respectively (39), thus the CDR3 α loop in particular may be capable of movement closer to the CDR1 α in the unbound A6 TCR and its mutants.

The fact that only $\alpha G28M$ and $\alpha S100T$ resulted in cooperativity, rather than the other tested pairs of mutations at those positions, seems to indicate a shift in interface architecture specific to those mutations which contradicts the notion of static modules that dictate

cooperativity between interface positions. In fact, a recent study of the TEM1-BLIP interface identified a pair of mutations (E104Y and Y105N on TEM1) that resulted in a backbone movement in BLIP and a rearrangement of the interface modules observed in the crystallized mutant complex (28). The α G28M mutation in particular has several notable characteristics, including that it has the highest association rate of the measured A6 TCR point mutations. The original energetic prediction for the $\Delta\Delta G$ of the point mutation α G28M was an outlier, where the affinity improvement was overestimated by over 1 kcal/mol (19). This may indicate that the structural modeling that was used to produce the α G28M prediction (which assumed a static backbone and neighboring side chains) was incorrect, but in the context of α S100T this conformation was achieved.

In addition to the cooperativity within individual chains, we also studied the degree of cooperativity when combining mutations from the different selection methods (Table 3), and rather than simple additivity found evidence of complex long-range interdependence. Particularly striking is that the combination of the high affinity mutations from each chain (**WFGMT-MSAQ**) yielded far less affinity than expected from the sum of $\Delta\Delta G$'s from each chain (**WFGMT** and **MSAQ**), a difference of 3.19 kcal/mol (200-fold K_D). Based on modeling of the α chain mutations that also show energetic and dissociation rate anticooperativity, it is possible that this is due to the mutations on each chain leading to hindrances to optimal binding. This is also supported by the fact that the level of energetic anticooperativity appears to diminish as the amount of mutations on the TCR α chain become smaller. It worth noting that the combination of D26W on the α chain and **MSAQ** on the β chain exhibits significant anticooperativity (0.97 kcal/mol), as those positions are separated by approximately 17 Å in the wild-type complex. This suggests possible shifts in the TCR docking mode or CDR movements due to either **MSAQ** or α D26W (or both) that can't be satisfied when combining these mutations together. While preliminary reports of the bound **MSAQ** mutant indicate CDR3 β loop and peptide residue Y5 side chain movement (45), it is unclear whether this occurs along with subtle α and β chain domain shifts as observed for the A6 TCR bound to a chemically modified Y5 peptide and MHC (38), or more localized loop movement as observed with the V7R peptide variant and MHC (16). Given the observed nonadditivity, it seems that the latter scenario is unlikely. A recently released structure of another TCR (MEL5), which shares the germ line CDR1 α and CDR2 α sequences with the A6 TCR (from the *TRAV12-2* gene), bound to a modified cancer epitope and HLA-A2 featured the same " α centric" binding mode of the TCR, with very similar CDR1 α conformation and antigen contacts, despite the considerable difference between the Tax and ELA peptides and the TCR CDR3 α loops and β chains (46). This supports the possibility that the mutant β chain (rather than the α chain) may undergo conformational change to allow the CDR1 α to retain its conserved binding mode, which even in the context of the CDR1 α mutants retains key binding residues such as α Q30.

These results can help to inform future rational design studies that combine multiple point mutations or multiple sets of mutations. As noted previously by Midelfort and Wittrup for combinations of mutations in a high affinity designed antibody (47), we found that distant, peripheral residue mutations can lead to additive binding energetics, as in the case of α S51M. However, nonadditivity is more likely when dealing with adjacent mutations, and mutations of adjacent residues to larger residues risks possible anticooperative effects (as observed when combining α D26W with α G28T or α G28M in this study). It is possible to rationally design cooperativity in neighboring residues, as performed by Lippow et al., however the authors noted that such multiple residue designs yield a lower success rate than point mutation design (8), likely due to the complexity and uncertainty at the atomic level of multiple modeled residues. Finally, combinations of a large set of neighboring mutations with distant residues can lead to nonadditivity, as we observed when combining the **MSAQ** and **MSAR** mutations on the β chain with various α chain mutations. The point mutant

β G101A, for example, may have led to better additivity with the single or multiple mutants of the α chain as its effects may be more localized than the larger sets of β chain mutations.

In summary, we have combined kinetic measurements of a large set of combinations of mutations with *in silico* structural modeling and analysis, leading to a clearer picture of the kinetic and structural basis of cooperativity and TCR/peptide/MHC binding. Future work in crystallization of high affinity mutants would help confirm whether loop movements and/or large-scale structural changes are responsible for cooperativity and anticooperativity within and among the chains. Additionally, further biophysical characterization of the mutants, using for instance isothermal calorimetry (ITC) or SPR with varying temperature points, would help to elucidate the entropic terms underlying the energetic and kinetic measurements of the TCR mutants presented in this study. The results presented here provide a dataset for further exploration of cooperativity and binding affinity analysis, and can help to guide future rational design and *in vitro* selection protein engineering efforts.

Supplementary Material

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Abbreviations

TCR	T cell receptor
HLA	human leukocyte antigen
MHC	major histocompatibility complex
CDR	complementarity determining region
SPR	surface plasmon resonance

REFERENCES

- (1). Wells JA. Additivity of mutational effects in proteins. *Biochemistry*. 1990; 29:8509–8517. [PubMed: 2271534]
- (2). Reichmann D, Rahat O, Albeck S, Meged R, Dym O, Schreiber G. The modular architecture of protein protein binding interfaces. *Proc Natl Acad Sci U S A*. 2005; 102:57–62. [PubMed: 15618400]
- (3). Kraich M, Klein M, Patino E, Harrer H, Nickel J, Sebald W, Mueller TD. A modular interface of IL-4 allows for scalable affinity without affecting specificity for the IL-4 receptor. *BMC Biol*. 2006; 4:13. [PubMed: 16640778]
- (4). del Sol A, Carbonell P. The modular organization of domain structures: insights into protein-protein binding. *PLoS Comput Biol*. 2007; 3:e239. [PubMed: 18069884]
- (5). Moza B, Buonpane RA, Zhu P, Herfst CA, Rahman AK, McCormick JK, Kranz DM, Sundberg EJ. Long-range cooperative binding effects in a T cell receptor variable domain. *Proc Natl Acad Sci U S A*. 2006; 103:9867–9872. [PubMed: 16788072]
- (6). Bernat B, Sun M, Dwyer M, Feldkamp M, Kossiakoff AA. Dissecting the binding energy epitope of a high-affinity variant of human growth hormone: cooperative and additive effects from

combining mutations from independently selected phage display mutagenesis libraries. *Biochemistry*. 2004; 43:6076–6084. [PubMed: 15147191]

- (7). Clark LA, Boriack-Sjodin PA, Eldredge J, Fitch C, Friedman B, Hanf KJ, Jarpe M, Liparoto SF, Li Y, Lugovskoy A, Miller S, Rushe M, Sherman W, Simon K, Van Vlijmen H. Affinity enhancement of an in vivo matured therapeutic antibody using structure-based computational design. *Protein Sci*. 2006; 15:949–960. [PubMed: 16597831]
- (8). Lippow SM, Wittup KD, Tidor B. Computational design of antibody-affinity improvement beyond in vivo maturation. *Nat Biotechnol*. 2007; 25:1171–1176. [PubMed: 17891135]
- (9). Sette A, Newman M, Livingston B, McKinney D, Sidney J, Ishioka G, Tangri S, Alexander J, Fikes J, Chesnut R. Optimizing vaccine design for cellular processing, MHC binding and TCR recognition. *Tissue Antigens*. 2002; 59:443–451. [PubMed: 12445314]
- (10). Lazoura E, Apostolopoulos V. Insights into peptide-based vaccine design for cancer immunotherapy. *Curr Med Chem*. 2005; 12:1481–1494. [PubMed: 15974981]
- (11). Boulter JM, Jakobsen BK. Stable, soluble, high-affinity, engineered T cell receptors: novel antibody-like proteins for specific targeting of peptide antigens. *Clin Exp Immunol*. 2005; 142:454–460. [PubMed: 16297157]
- (12). Miles JJ, Silins SL, Burrows SR. Engineered T cell receptors and their potential in molecular medicine. *Curr Med Chem*. 2006; 13:2725–2736. [PubMed: 17073624]
- (13). Ashfield R, Jakobsen BK. Making high-affinity T-cell receptors: a new class of targeted therapeutics. *IDrugs*. 2006; 9:554–559. [PubMed: 16871464]
- (14). Richman SA, Kranz DM. Display, engineering, and applications of antigen-specific T cell receptors. *Biomol Eng*. 2007; 24:361–373. [PubMed: 17409021]
- (15). Garboczi DN, Ghosh P, Utz U, Fan QR, Biddison WE, Wiley DC. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature*. 1996; 384:134–141. [PubMed: 8906788]
- (16). Ding YH, Baker BM, Garboczi DN, Biddison WE, Wiley DC. Four A6-TCR/peptide/HLA-A2 structures that generate very different T cell signals are nearly identical. *Immunity*. 1999; 11:45–56. [PubMed: 10435578]
- (17). Davis-Harrison RL, Armstrong KM, Baker BM. Two different T cell receptors use different thermodynamic strategies to recognize the same peptide/MHC ligand. *J Mol Biol*. 2005; 346:533–550. [PubMed: 15670602]
- (18). Armstrong KM, Baker BM. A comprehensive calorimetric investigation of an entropically driven T cell receptor-peptide/major histocompatibility complex interaction. *Biophys J*. 2007; 93:597–609. [PubMed: 17449678]
- (19). Haidar JN, Pierce B, Yu Y, Tong W, Li M, Weng Z. Structure-based design of a T cell receptor leads to nearly 100 fold improvement in binding affinity for pepMHC. *Proteins*. 2009; 74:948–960. [PubMed: 18767161]
- (20). Li Y, Moysey R, Molloy PE, Vuidepot AL, Mahon T, Baston E, Dunn S, Liddy N, Jacob J, Jakobsen BK, Boulter JM. Directed evolution of human T-cell receptors with picomolar affinities by phage display. *Nat Biotechnol*. 2005; 23:349–354. [PubMed: 15723046]
- (21). Garboczi DN, Utz U, Ghosh P, Seth A, Kim J, VanTienhoven EA, Biddison WE, Wiley DC. Assembly, specific binding, and crystallization of a human TCR-alpha-beta with an antigenic Tax peptide from human T lymphotropic virus type 1 and the class I MHC molecule HLA-A2. *J Immunol*. 1996; 157:5403–5410. [PubMed: 8955188]
- (22). Garboczi DN, Hung DT, Wiley DC. HLA-A2-peptide complexes: refolding and crystallization of molecules expressed in *Escherichia coli* and complexed with single antigenic peptides. *Proc Natl Acad Sci U S A*. 1992; 89:3429–3433. [PubMed: 1565634]
- (23). Rich RL, Myszkowski DG. Advances in surface plasmon resonance biosensor analysis. *Curr Opin Biotechnol*. 2000; 11:54–61. [PubMed: 10679342]
- (24). Kortemme T, Baker D. A simple physical model for binding energy hot spots in protein-protein complexes. *Proc Natl Acad Sci U S A*. 2002; 99:14116–14121. [PubMed: 12381794]
- (25). Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE. The Protein Data Bank. *Nucleic Acids Res*. 2000; 28:235–242. [PubMed: 10592235]

- (26). Lazaridis T, Karplus M. Effective energy function for proteins in solution. *Proteins-Structure Function and Genetics*. 1999; 35:133–152.
- (27). Zhang C, Vasmataz G, Cornette JL, DeLisi C. Determination of atomic desolvation energies from the structures of crystallized proteins. *J Mol Biol*. 1997; 267:707–726. [PubMed: 9126848]
- (28). Reichmann D, Cohen M, Abramovich R, Dym O, Lim D, Strynadka NC, Schreiber G. Binding hot spots in the TEM1-BLIP interface in light of its modular architecture. *J Mol Biol*. 2007; 365:663–679. [PubMed: 17070843]
- (29). Potapov V, Reichmann D, Abramovich R, Filchitski D, Zohar N, Ben Halevy D, Edelman M, Sobolev V, Schreiber G. Computational redesign of a protein-protein interface for high affinity and binding specificity using modular architecture and naturally occurring template fragments. *J Mol Biol*. 2008; 384:109–119. [PubMed: 18804117]
- (30). Wu LC, Tuot DS, Lyons DS, Garcia KC, Davis MM. Two-step binding mechanism for T-cell receptor recognition of peptide MHC. *Nature*. 2002; 418:552–556. [PubMed: 12152083]
- (31). Harrison, R. L. Davis; Insaiddoo, FK.; Baker, BM. T cell receptor binding transition states and recognition of peptide/MHC. *Biochemistry*. 2007; 46:1840–1850. [PubMed: 17249694]
- (32). Laugel B, Boulter JM, Lissin N, Vuidepot A, Li Y, Gostick E, Crotty LE, Douek DC, Hemelaar J, Price DA, Jakobsen BK, Sewell AK. Design of soluble recombinant T cell receptors for antigen targeting and T cell inhibition. *J Biol Chem*. 2005; 280:1882–1892. [PubMed: 15531581]
- (33). Stone JD, Chervin AS, Kranz DM. T-cell receptor binding affinities and kinetics: impact on T-cell activity and specificity. *Immunology*. 2009; 126:165–176. [PubMed: 19125887]
- (34). Albeck S, Schreiber G. Biophysical characterization of the interaction of the beta-lactamase TEM-1 with its protein inhibitor BLIP. *Biochemistry*. 1999; 38:11–21. [PubMed: 9890878]
- (35). Armstrong KM, Insaiddoo FK, Baker BM. Thermodynamics of T-cell receptor-peptide/MHC interactions: progress and opportunities. *J Mol Recognit*. 2008; 21:275–287. [PubMed: 18496839]
- (36). Borg NA, Ely LK, Beddoe T, Macdonald WA, Reid HH, Clements CS, Purcell AW, Kjer Nielsen L, Miles JJ, Burrows SR, McCluskey J, Rossjohn J. The CDR3 regions of an immunodominant T cell receptor dictate the ‘energetic landscape’ of peptide-MHC recognition. *Nat Immunol*. 2005; 6:171–180. [PubMed: 15640805]
- (37). Weber KS, Donermeyer DL, Allen PM, Kranz DM. Class II restricted T cell receptor engineered in vitro for higher affinity retains peptide specificity and function. *Proc Natl Acad Sci U S A*. 2005; 102:19033–19038. [PubMed: 16365315]
- (38). Gagnon SJ, Borbulevych OY, Davis-Harrison RL, Turner RV, Damirjian M, Wojnarowicz A, Biddison WE, Baker BM. T cell receptor recognition via cooperative conformational plasticity. *J Mol Biol*. 2006; 363:228–243. [PubMed: 16962135]
- (39). Armstrong KM, Piepenbrink KH, Baker BM. Conformational changes and flexibility in T-cell receptor recognition of peptide-MHC complexes. *Biochem J*. 2008; 415:183–196. [PubMed: 18800968]
- (40). Gakamsky DM, Luescher IF, Pecht I. T cell receptor-ligand interactions: a conformational preequilibrium or an induced fit. *Proc Natl Acad Sci U S A*. 2004; 101:9063–9066. [PubMed: 15178754]
- (41). Schreiber G. Kinetic studies of protein-protein interactions. *Curr Opin Struct Biol*. 2002; 12:41–47. [PubMed: 11839488]
- (42). Harel M, Cohen M, Schreiber G. On the dynamic nature of the transition state for protein-protein association as determined by double-mutant cycle analysis and simulation. *J Mol Biol*. 2007; 371:180–196. [PubMed: 17561113]
- (43). Frisch C, Fersht AR, Schreiber G. Experimental assignment of the structure of the transition state for the association of barnase and barstar. *J Mol Biol*. 2001; 308:69–77. [PubMed: 11302708]
- (44). Clements CS, Dunstone MA, Macdonald WA, McCluskey J, Rossjohn J. Specificity on a knife-edge: the alphabeta T cell receptor. *Curr Opin Struct Biol*. 2006; 16:787–795. [PubMed: 17011774]
- (45). Sami M, Rizkallah PJ, Dunn S, Molloy P, Moysey R, Vuidepot A, Baston E, Todorov P, Li Y, Gao F, Boulter JM, Jakobsen BK. Crystal structures of high affinity human T cell receptors

bound to peptide major histocompatibility complex reveal native diagonal binding geometry. *Protein Eng Des Sel.* 2007; 20:397–403. [PubMed: 17644531]

- (46). Cole DK, Yuan F, Rizkallah PJ, Miles JJ, Gostick E, Price DA, Gao GF, Jakobsen BK, Sewell AK. Germ line governed recognition of a cancer epitope by an immunodominant human T-cell receptor. *J Biol Chem.* 2009; 284:27281–27289. [PubMed: 19605354]
- (47). Midelfort KS, Wittrup KD. Context-dependent mutations predominate in an engineered high-affinity single chain antibody fragment. *Protein Sci.* 2006; 15:324–334. [PubMed: 16434745]

Figure 1.

Positions of TCR mutations on the structure of the wild-type TCR/peptide/MHC complex (PDB code 1AO7) from the side (A) and top (B; peptide and MHC not shown). TCR α chain is shown in red, TCR β chain in green, MHC in yellow, Tax peptide in magenta, and mutated residues in blue and slate. In the right panel, residues mutated in this study are labeled by their wild-type amino acid code and position, and inter-residue distances are shown with dotted lines.

Figure 2.

Sensorgrams for Tax/HLA-A2 binding to wild-type A6 TCR and mutants. In each experiment, approximately 400 Response Units (RU) of TCR was immobilized per cell and gradients of Tax/HLA-A2 were injected over the cells using serial two-fold dilutions. Lines representing global fits of the data to the Langmuir 1:1 model are shown, and residuals are given below the sensorgrams. Tax/HLA-A2 concentrations shown are: 0.84 μ M, 0.42 μ M, 0.21 μ M (A6 wild-type, **DRIST**); 0.45 μ M, 0.225 μ M, 0.1125 μ M (**DFTMT**); 0.42 μ M, 0.21 μ M, 0.115 μ M (**DWTMT**); 0.675 μ M, 0.3375 μ M, 0.16875 μ M (G28T **MSAR**, D26W-**MSAQ**).

Figure 3.

Cooperative $\Delta\Delta G$ for combinations of TCR mutations, organized according to chain containing the mutations. Cooperative $\Delta\Delta G$ is defined as the difference between measured $\Delta\Delta G$ and the sum of $\Delta\Delta G$ from component point mutations measured separately.

Figure 4. Additive versus measured binding energy change for A) total energy ($\Delta\Delta G$), B) association rate energy ($\Delta\Delta G_{on}$), and C) dissociation rate energy ($\Delta\Delta G_{off}$) for 23 measured TCR mutations. Point types indicate the TCR chain containing the mutations, with $\lambda = \alpha$ chain only, $\sigma = \beta$ chain only, $\nu = \alpha$ and β chains. The dashed line represents additive binding energetics. Correlations are: A) 0.56 (all points), 0.47 (α chain only) B) 0.46 (all points), 0.66 (α chain only), C) 0.87 (all points), 0.82 (α chain

Figure 5. Structural models of mutated TCR α chain residues 26 and 28 in mutants **WRMMT** (A) and **WRTMT** (B), produced by Rosetta (24). TCR α chain is red, TCR β chain is green, peptide is magenta, MHC is yellow, and mutant residues at α 26 and α 28 are blue. Shortest inter-residue heavy atom distances are denoted by dotted lines.

Table 1

Measured kinetic results for TCR mutants binding peptide/MHC.

Mutant *	k_{on} , $\times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	k_{off} , $\times 10^{-3} \text{ s}^{-1}$	K_D , $\times 10^{-7} \text{ M}$
A6 wild-type	5.1 ± 0.5	108 ± 14	21 ± 3.5
α chain			
DRISA	4.6 ± 0.4	52 ± 0.7	11 ± 1.0
DRIST	2.2 ± 0.3	10 ± 0.2	4.6 ± 0.5
DRLSA	7.9 ± 0.7	70 ± 5.2	8.9 ± 1.0
DRLST	3.7 ± 0.3	14 ± 0.4	3.7 ± 0.3
DRMSA	8.0 ± 0.3	43 ± 2.5	5.4 ± 0.4
DRMST	13.8 ± 3.1	15 ± 1.6	1.1 ± 0.3
DRMMT	11.2 ± 1.4	6.6 ± 0	5.058 ± 0.09
DRTMT	2.7 ± 0.1	3.8 ± 0	0.614 ± 0.07
DFMMT	10.6 ± 0.5	11 ± 0.3	1.0 ± 0.06
DFTMT	2.3 ± 0.1	3.3 ± 0	0.914 ± 0.08
WFGMT	6.3 ± 0.3	1.4 ± 0	0.3021 ± 0.01
WRMMT	2.1 ± 0.8	7.2 ± 1	2.34 ± 1.4
WRTMT	1.1 ± 0.3	3.0 ± 0	2.26 ± 0.7
WFTMT	2.0 ± 0.7	4.8 ± 0	1.24 ± 0.9
β chain			
AGAQ	2.1 ± 0.4	3.2 ± 0	3.15 ± 0.3
MSGR	2.8 ± 0.8	10 ± 0.8	3.6 ± 1.0
MSAR	3.6 ± 0.1	0.19 ± 0	0.053 ± 0.02
MSAQ	4.2	0.12	0.028
$\alpha+\beta$ chain			
G28T-MSAR	3.2 ± 0.7	0.10 ± 0.01	0.031 ± 0.007
G28T-MSAQ	5.0 ± 0.5	0.13 ± 0.05	0.026 ± 0.01
D26W-MSAQ	1.9 ± 0.1	0.045 ± 0.01	0.023 ± 0.007
WFGMS-MSAQ	3.3 ± 0.1	0.13 ± 0.02	0.04 ± 0.007
WFGMT-MSAQ	1.9 ± 0.4	0.11 ± 0.03	0.062 ± 0.02

* Amino acid sequence of mutant, with mutated residues in bold. For α chain mutations, residues 26-28, 51, and 100 are indicated, and for β chain mutations, residues 99-102.

Errors calculated using the sample standard deviation of the three measured gradients. For mutant **MSAQ** no standard deviation was obtained due to lack of replicate measurements.

Results for A6 wild-type, **WFGMT** and **WFTMT** reported previously by Haidar et al. (19).

Table 2

Binding energies and cooperativity for measured TCR mutations.

Mutant	CDR1 α				CDR2 α				CDR3 β				Measured Binding			Cooperativity ^J		
	$\alpha 26$	$\alpha 27$	$\alpha 28$	$\alpha 51$	$\alpha 100$	$\beta 99$	$\beta 100$	$\beta 101$	$\beta 102$	$\Delta\Delta G$	$\Delta\Delta G_{on}$	$\Delta\Delta G_{off}$	$\Delta\Delta G$	$\Delta\Delta G_{on}$	$\Delta\Delta G_{off}$	$\Delta\Delta G$	$\Delta\Delta G_{on}$	$\Delta\Delta G_{off}$
DRISA	D	R	I	S	A	A	G	G	R	-0.38	0.06	-0.44	-0.11	-0.10	-0.01	-0.10	-0.02	-0.01
DRIST	D	R	I	S	T	A	G	G	R	-0.91	0.49	-1.40	-0.16	-0.02	-0.14	-0.16	-0.02	-0.14
DRLSA	D	R	L	S	A	A	G	G	R	-0.51	-0.26	-0.25	-0.04	-0.13	0.09	-0.04	-0.13	0.09
DRLST	D	R	L	S	T	A	G	G	R	-1.03	0.20	-1.23	-0.08	-0.02	-0.06	-0.08	-0.02	-0.06
DRMSA	D	R	M	S	A	A	G	G	R	-0.81	-0.26	-0.55	-0.29	0.00	-0.29	-0.29	0.00	-0.29
DRMST	D	R	M	S	T	A	G	G	R	-1.77	-0.59	-1.18	-0.77	-0.68	-0.09	-0.77	-0.68	-0.09
DRMMT	D	R	M	M	T	A	G	G	R	-2.12	-0.46	-1.66	-0.75	-0.52	-0.23	-0.75	-0.52	-0.23
DRTMT	D	R	T	M	T	A	G	G	R	-1.62	0.37	-1.99	0.01	0.17	-0.16	0.01	0.17	-0.16
DFMMT	D	F	M	M	T	A	G	G	R	-1.78	-0.43	-1.35	-0.21	-0.22	0.01	-0.21	-0.22	0.01
DFTMT	D	F	T	M	T	A	G	G	R	-1.61	0.46	-2.07	0.24	0.53	-0.29	0.24	0.53	-0.29
WFGMT	W	F	G	M	T	A	G	G	R	-2.72	-0.13	-2.59	-0.57	-0.67	0.10	-0.57	-0.67	0.10
WRMMT	W	R	M	M	T	A	G	G	R	-1.08	0.52	-1.60	1.36	0.02	1.34	1.36	0.02	1.34
WRTMT	W	R	T	M	T	A	G	G	R	-1.23	0.89	-2.12	1.47	0.25	1.22	1.47	0.25	1.22
WFTMT	W	F	T	M	T	A	G	G	R	-1.28	0.56	-1.84	1.64	0.19	1.45	1.64	0.19	1.45
AGAQ	D	R	G	S	S	A	G	A	Q	-1.56	0.52	-2.08	-0.92	-0.56	-0.36	-0.92	-0.56	-0.36
MSGR	D	R	G	S	S	M	S	G	R	-1.04	0.36	-1.40	-0.42	-0.27	-0.15	-0.42	-0.27	-0.15
MSAR	D	R	G	S	S	M	S	A	R	-3.54	0.21	-3.75	-1.83	-1.02	-0.81	-1.83	-1.02	-0.81
MSAQ	D	R	G	S	S	M	S	A	Q	-3.93	0.11	-4.04	-2.67	-1.60	-1.07	-2.67	-1.60	-1.07
G28T-MSAR	D	R	T	S	S	M	S	A	R	-3.87	0.27	-4.14	-1.39	-0.79	-0.60	-1.39	-0.79	-0.60
G28T-MSAQ	D	R	T	S	S	M	S	A	Q	-3.97	0.01	-3.98	-1.94	-1.53	-0.41	-1.94	-1.53	-0.41
D26W-MSAQ	W	R	G	S	S	M	S	A	Q	-4.04	0.57	-4.61	-1.71	-1.58	-0.13	-1.71	-1.58	-0.13
WFGMS-MSAQ	W	F	G	M	S	M	S	A	Q	-3.71	0.26	-3.97	-0.79	-1.59	0.80	-0.79	-1.59	0.80
WFGMT-MSAQ	W	F	G	M	T	M	S	A	Q	-3.46	0.60	-4.06	-0.05	-1.65	1.60	-0.05	-1.65	1.60

^J Cooperativity equals difference between measured $\Delta\Delta G$ for the mutant and the sum of $\Delta\Delta G$ for the component point mutations.

Mutant residues are shown in bold, and mutants with cooperative or anticoperative energetics (cooperative $\Delta\Delta G < -0.5$ kcal/mol or > 0.5 kcal/mol) shown in gray shading.

All $\Delta\Delta G$ values given in units of kcal/mol. $\Delta\Delta G_{on} = RT \times \ln(k_{on\_wild-type}/k_{on\_mutant})$, and $\Delta\Delta G_{off} = RT \times \ln(k_{off\_mutant}/k_{off\_wild-type})$, with k_{on} and k_{off} equal to the association and dissociation rates, respectively. $\Delta\Delta G = RT \times \ln(k_{off\_mutant}/k_{on\_mutant}) = \Delta\Delta G_{on} + \Delta\Delta G_{off}$.

Table 3

Nonadditive energetics between mutant TCR chains.

Mutant	Association Rate				Dissociation Rate				Total	
	$\Delta\Delta G_{on}$	Sum ¹	IC_Coop ²	$\Delta\Delta G_{off}$	Sum ¹	IC_Coop ²	$\Delta\Delta G$	Sum ¹	IC_Coop ²	
G28T-MSAR	0.27	0.04	0.23	-4.14	-4.35	0.21	-3.87	-4.30	0.43	
G28T-MSAQ	0.01	-0.06	0.07	-3.98	-4.64	0.66	-3.97	-4.69	0.72	
D26W-MSAQ	0.57	0.55	0.02	-4.61	-5.55	0.94	-4.04	-5.01	0.97	
WFGMT-MSAQ	0.60	-0.02	0.62	-4.06	-6.63	2.57	-3.46	-6.65	3.19	

¹ Sum of the $\Delta\Delta G$ from the mutant α chain and mutant β chain.

² Inter-chain cooperativity, defined as the difference between the measured $\Delta\Delta G$ and the sum of measured $\Delta\Delta G$ s from each mutant TCR chain.

All values are in units of kcal/mol