STRUCTURE GUIDED DESIGN AND COMPUTATIONAL SCREENING IN CELLULAR IMMUNITY

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Abstract

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Proteins are often engineered for greater affinity and/or specificity to achieve therapeutic benefit [1]. For example, as antibodies and T cell receptors (TCRs) have emerged as a new class of therapeutics, phage and yeast display libraries have been used to affinity mature these molecules for specific targets. Although effective, these techniques offer limited structural control of the engineered sites under given selection pressures, and are often termed “irrational design” [2]. In contrast, in silico methods use structure-based physical models to estimate interaction energies, allowing for “rational” design and more targeted interactions.
The role of the TCR in identifying immunological targets and signaling appropriate responses has resulted in exciting translational opportunities. Yet TCRs mediate one of the most complex protein-protein interactions in biology, with intricate biochemical/biomolecular mechanisms intersecting structure, affinity, and specificity. Here, we address these hallmarks of TCR recognition, emphasizing the use of computational models and structure guided design to examine the fine details of TCR interfaces. With broad implications to T cell based immunotherapies, we highlight how the ‘imperfect’ interfaces of TCRs bound to their ligands influences aspects of TCR design, T cell specificity, and epitope recognition of the immune system.
I would like to dedicate this thesis to my wife, Sarah C. Riley, whose countless sacrifices, unending patience, and unconditional love has given me every chance to succeed.
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T cell recognition of diseased cells underlies cellular immunity. Recognition is facilitated by the T cell receptor (TCR), a complex sensor and signaling protein whose biology and biochemistry has been intensely studied as immunotherapies for cancer, infectious disease, and autoimmunity have progressed from theoretical considerations to practical implementation. Cellular immunity is dependent upon effective discrimination between foreign and self-antigens, and obtaining an accurate depiction of TCR recognition mechanisms is a critical step towards discovering advances in vaccine design and cancer immunotherapies.
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CHAPTER 1:

INTRODUCTION

1.1 T Cell Mediated Immunity

In vertebrates, the adaptive immune response serves as a pathway for the identification and destruction of foreign pathogens. The two major cellular entities of the adaptive immune system are B and T cells, which originate from lymphocytes within the bone marrow and thymus, respectively. Despite their biological similarities, B cells and T cells differ in key ways. B cells release antibodies elicited against targets of seemingly unlimited structural and chemical diversity directly into the environment, whereas T cells utilize cell surface-bound T cell receptors (TCRs) restricted to linear peptide antigens presented by major histocompatibility complex (MHC) proteins. All nucleated cells express MHC molecules on the cell surface, which present peptide fragments derived from intracellular proteins (Figure 1.1). These peptides are normally derived from normal proteins involved in cellular processes, but abnormal peptides derived from bacterial, viral, or cancerous proteins may also be presented [4]. T cells via their expressed TCRs are responsible for monitoring cell surface MHC molecules and eliminating cells presenting abnormal peptides. Thus, TCRs have the remarkable task of discriminating
abnormal from normal peptides in context of a self-entity (MHC protein). This phenomenon is termed MHC restriction and is a fundamental feature of T cell biology.

1.2 T Cell Diversity and Thymic Selection

Although T cells and TCRs are MHC restricted, significant variability is required for adequate protection of the host organism. To defend against the almost limitless number of antigenic peptides that are chemically and structurally distinct, TCRs are a product of somatic gene recombination occurring during T cell development in the thymus. By the random selection of many available variable (V), diversity (D), and joining (J) genes, a developing T cell generates two polypeptide chains. These α and β chains combine to form a heterodimeric TCR almost certainly unique from other randomly recombined TCRs.

The process of V(D)J recombination results in three antigen complementarity-determining regions (CDR) for each α and β TCR chain. The variable gene segment directly encodes for the first two CDRs (germline CDR), while the third CDR (hypervariable CDR) is located at the junction of gene segments. This junction encompasses the largest amount of sequence variation, where additional nucleotide insertions or deletions can occur due to the imprecise recombination process. Thus, the immune system generates a highly diverse TCR repertoire to respond to the vast universe of antigenic peptides.

The creation of such a vast and seemingly random repertoire of TCRs requires the immune system to impose additional regulations on developing T cells to maintain MHC
Figure 1.1 *Antigen processing and presentation of the class I MHC complex.* The proteasome degrades intracellular proteins and the resulting peptide fragments are transported into the Endoplasmic Reticulum (ER). Peptide fragments are loaded onto class I major histocompatibility complexes (MHC) and transported to the cell surface. Cytotoxic T cells specifically recognize peptides presented by class I MHC molecules, via the T cell receptor (TCR).
restriction. As T cells develop within the thymus, they undergo selection to remove T cells that strongly respond to peptides derived from the host organism, or those that lack a basal recognition for the MHC molecule. This process eliminates approximately 99.9% of T cells [5], but the remaining T cell repertoire is thus only capable of recognizing a ‘non-self’ peptide in context of the MHC molecule.

1.3 Structural Properties of TCR/pMHC Complexes

To understand the guiding principles driving TCR recognition and MHC restriction, the structural properties of these molecules provide the necessary insight. From the perspective of a TCR, peptides lie relatively flat in the MHC binding groove, flanked closely by the MHC α-helices. This is a defining feature of peptide/MHC complexes, as TCRs must recognize a composite surface to bind and initiate an immune response. To engage this composite surface, αβ TCRs form a highly conserved tertiary structure resembling the Fab fragment of an antibody (Figure 1.2A). Within this fold, the six CDRs of the TCR form loops, thus generating a unique surface capable of sampling distinct peptide features in context of the MHC surface. Early structural studies confirmed both the peptide and the MHC play pivotal roles in TCR recognition, demonstrating that TCRs often bury significant amounts of surface area spanning both the peptide and MHC [6]. Several generalizations arose from these early structures, the most notable of which assumed the germline encoded CDR loops were responsible for recognition of the MHC protein and the more diverse hypervariable loops were responsible for recognition of the peptide [7].
Figure 1.2 **Structural properties of a TCR in complex with a peptide/MHC.** The orientation of the conventional αβ A6 TCR in complex with the HTLV-1 TAX peptide presented by the class I MHC molecule HLA-A2:01 shown in A. (B) A 90° rotation over the binding interface demonstrates the composite surface TCR CDR loops engage. PDB: 1QRN
generalization stemmed from observations where the germline encoded loops docked predominately along the α-helices of the MHC protein (leading to MHC restriction), whereas the hypervariable loops engaged the peptide (yielding antigen specificity) (Figure 1.2B).

Many subsequent studies continued to define and deconstruct the “roles” of the various components of the TCR interface. The outcome of almost two decades of work has concluded that, despite the appeal of a generalized model, the fundamental origins of MHC restriction and TCR recognition are far more complex than previously envisioned. It is now clear that, although hypervariable loops most commonly engage the peptide, germline loops (particularly the CDR1 loops) also frequently interact with the peptide and can contribute significantly to specificity [8]. Overall, when considering how TCRs engage their ligand, one must consider the composite surface composed of the peptide and the MHC protein, and all six TCR CDR loops (and sometimes elements outside of the loops). Taken together, the rules we might envision are likely to be bent or broken in any individual case.

1.4 TCR binding affinity, specificity, and the relationship to T cell functional activity

Understanding the principles of TCR recognition and these structure-function relationships is of paramount importance in today’s age of autologous cell therapy. Pioneering studies from the 1980s to the early 2000s demonstrated that T cell specificity could be redirected via the transfer of TCR α and β genes [9-11] (Figure 1.3). This led to
Figure 1.3 Adoptive Cell Transfer Presents an Alternative to Cancer Treatment. Traditionally, tumor infiltrating lymphocytes (TIL) are removed from a tumor sample, activated and expanded \textit{ex vivo}, before reintroduction to the patient. Genetically engineering T cells via transfer of TCR α and β genes allow for the generation of cancer specific T cells outside of the process of positive and negative selection.
the proposal for TCR-based gene therapy of cancer [12], and was followed by several studies demonstrating how anti-viral and anti-tumor immunity could be generated via gene-engineered T cells and adoptive cell transfer[13-16].

Early findings found that T cell functional responses could be correlated with receptor binding affinity [17-21], consistent with experiences with other biologic-based therapies (e.g., monoclonal antibodies, receptor antagonists, etc.) [22]. However, in a seeming paradox, native TCRs maintain relatively moderate affinity for their ligands, typically in the single to double digit micromolar range [23]. Low binding affinities are often associated with “imperfect” protein-ligand interfaces [24], and the low shape complementarity, gaps, uncompensated charges, and entropic barriers resulting from CDR loop motions of TCR/pMHC interfaces characterize them as such [25-30]. Although these imperfect interfaces observed with native TCR binding events may seem counterintuitive compared to other immunological receptors (such as antibodies), the naturally weak affinity of TCRs may have biological implications. For example, humans have a fixed number of TCRs estimated to be in the tens of millions [31], which results in a built in requirement for TCR cross-reactivity as the theoretical number of possible peptide antigens is many orders of magnitude larger [32, 33] (Figure 1.4). From this perspective, it is tempting to associate the weak binding and imperfect interfaces of TCRs with their penchant for cross reacting with multiple ligands. However, yet other examples have shown the existence of an affinity ‘threshold,’ where improvements in TCR affinity did not lead to improvements in specificity for specific antigens and in actuality decreased functional T cell responses [20]. Although the biological mechanisms for this are unclear,
Figure 1.4 The possible antigen universe far exceeds the estimated T cell repertoire. Approximately $10^7$ clonally distinct T cells circulate the body, which is orders of magnitude lower than possible peptide permutations.
these studies highlight the importance of considering the molecular mechanisms of TCR recognition for the development of immunotherapies.

1.5 Identifying and Engineering the ‘Optimal’ TCR

As alluded to above, current studies acknowledge that clinically useful TCRs require a degree of both affinity and specificity for the target antigen. Recent findings have shown that careful control is necessary when modifying TCRs, as affinity and specificity are not mutually exclusive. Due to their cross-reactive nature, enhancing affinity may introduce new reactivities: improving affinity against one antigen can improve affinity towards others, leading to reactivity otherwise ignored by T cells expressing the wild-type receptor. This could include self-antigens, leading to possible off-target recognition. Such an outcome is believed to have led to fatal autoimmunity in a recent clinical trial targeting the MAGE-A3 melanoma antigen [34]. This is especially true considering the composite peptide:MHC surface of TCR ligands, as improving affinity indiscriminately can lead to a TCR with improved recognition of the ubiquitous MHC molecule, regardless of the presented peptide. Thus, such approaches can conceptually decrease peptide specificity.

The most common strategy to improve TCRs utilizes traditional irrational protein engineering techniques, best known as directed evolution (Figure 1.5). Following similar principles as Darwinian evolution, TCRs are randomly mutated, often through error prone PCR or degenerate primers, and expressed on the surface of phage/yeast cells. From here,
Figure 1.5 Schematic of Directed Evolution for TCRs. The cycle begins by generating a pool of yeast cells expressing a randomized TCR gene library. Applying a selection pressure, such as binding to a target pMHC, and discarding cells expressing TCRs not conforming to the selection pressure allow for rapid isolation of desired biophysical properties.
the application of a selection pressure (usually the target antigen of interest) allows for screening TCRs for a number of biophysical properties, although stability of the surface expressed receptor also tends to bias designs [35]. These approaches, while successful with monoclonal antibodies and other biologics, are random by design and incapable of addressing the dual peptide/MHC nature of TCR specificity. Indeed, the blind affinity enhancements that emerge from irrational design can exacerbate the issue, as seen in the aforementioned clinical trial with an affinity enhanced TCR targeting the MAGE-A3 melanoma antigen. Patients treated with T cells engineered to express these modified TCRs unfortunately suffered from fatal cardiovascular toxicity. Jakobsen et al. later found these TCRs recognized an unrelated peptide derived from titin, and emphasized the need for improved methods to define the peptide specificity of TCRs before therapeutic use [36].

To address some of the shortcomings of a directed evolution engineering strategy, rational structure guided design offers a targeted approach to designing TCRs. By utilizing structural information throughout the protein engineering process, the introduction of targeted mutations allows for fine manipulation of biophysical properties. The complexities of TCR/pMHC interfaces are naturally suited for targeted mutations, but many limitations remain for rational design of TCRs to become widely adopted. Most notably, the large and complex binding interfaces of TCRs are notoriously difficult to predict, model, and assign energetic values to. This can lead to downstream effects, such as poorly modeled hydrogen bonds and electrostatics, which require precise distance and geometric orientations to be favorable and offset the unfavorable desolvation
penalty. As evidence of this, many rational design attempts of generic proteins have led to a general replacement of small and/or polar residues for large and hydrophobic mutations [37]. The significance of this is unclear: while increasing buried surface area is a well-known strategy for enhancing binding, it is not dependent on the precise geometric restraints required for favorable electrostatic interactions and may contribute to a loss in specificity. In contrast, electrostatic contributions to affinity can vary widely due to high desolvation penalties. Considering these shortcomings of both rational and irrational design, the optimal approach to designing TCRs with the constraints of current technology likely requires elements of both to fully capitalize on their distinct advantages.

1.6 Defining TCR Peptide Specificity

Although developments in protein engineering allowed for improvements and optimization of TCR affinity, defining and controlling specificity remains problematic due to the unique specifications TCRs must have to engage the composite peptide/MHC surface. Defining levels of specificity/cross-reactivity is of crucial importance when assessing TCRs for clinical use. Many assessments assume molecular mimicry and simply examine small collections of single amino acid peptide variants, reporting on “fine specificity” rather than the repertoire of ligands actually recognized [19]. Short of direct evaluation in a living organism, the most effort- and time-consuming approach to define TCR specificity is to measure T cell responses in the presence of numerous cell and tissue samples. This approach conclusively identified cross-reactivity in the clinical trial with the
aforementioned MAGE-A3-targeted TCR [36], and has the capacity to directly identify biologically relevant cross-reactive peptides. However, it is not currently feasible or practical to test all tissue samples within all stages of development.

A more direct method to assess TCR specificity and cross-reactivity utilizes pooled, chemically synthesized peptide libraries. These libraries of \( \sim 10^{12} \) peptides are loaded onto antigen presenting cells and T cells expressing a single receptor screened for functional responses [38]. An alternative method more limited to binding specificity utilizes a gene construct linking the peptide to the MHC protein through a flexible protein linker, providing a template for random mutagenesis followed by yeast display and identification of TCR-recognized peptides via flow cytometry and sequencing [39]. Both of these approaches have limitations, but permit interpolation of the number of and type of peptides recognized. Although in both cases the number of possible peptides outnumbers those assessed experimentally, these might be the best technologies capable of illustrating changes in specificity. Additionally, as our understanding of the structural properties of these molecules grow, computational modeling based on structural information may be able to supplement these experimental approaches.

1.7 Structural features of pMHC molecules for novel immunotherapies

Although adoptive cell transfer emphasizing the T cell and TCR has shown remarkable promise for targeting cancer specific antigens, logistical and manufacturing hurdles present an obstacle to widespread adoption. An alternative branch of
immunotherapy aims to stimulate the immune system through use of a ‘peptide vaccine.’ Instead of isolating and engineering T cells for tumor-targeting ability, this therapy aims to treat patients with a vaccine designed to mimic novel epitopes generated by spontaneous cancer mutations (neoepitopes) and stimulate cytotoxic T cells. Unlike vaccination against viruses, where the main objective is to stimulate B cells and antibody production, peptide vaccines require additional considerations. Namely, the epitope used within the vaccine must first be able to associate with the MHC molecule. Class I MHC molecules have distinct epitope preferences and typically bind peptides 9-11 amino acids long (Figure 1.6A) [3]. Additionally, each MHC polymorphism prefers certain ‘anchor’ residues in a companion peptide epitope (Figure 1.6B) [40]. These simple constraints have led to a variety of epitope prediction servers to facilitate and predict epitopes bound and presented by various MHC polymorphisms in silico [41].

The aforementioned epitope prediction servers coupled with various experimental methods have shown exciting promise identifying immunogenic epitopes [42]. Conceptually, if a neoepitope contains a mutation at a preferred anchor position, and therefore improving the affinity of the neoepitope for the MHC molecule, the resulting antigen may be sufficiently distinct from self-antigens to where T cells will not have been tolerized to its existence in the thymus.

However, to initiate an immune response, a potential epitope must have features beyond the ability to bind adequately to an MHC molecule [43]. Although targeting immunogenic neoepitopes that contain favorable so-called ‘anchor’ residues is clearly
Figure 1.6 **The peptide length preferences of a class I MHC molecule.** (A) A peptide associated with HLA-A2 is most likely to be 9 residues long. (B) Sequence comparisons of 2850 nonameric peptides associated with HLA-A2 have a clear preference for select hydrophobic residues in positions 2 and 9, with greater diversity elsewhere. Data adapted from [3].
required, other factors can initiate an immune response, such as structural perturbations and chemical utility of the unique mutation. The randomness of T cell receptor recombination followed by positive and negative selection would suggest that, hypothetically, there exists a T cell and a TCR capable of recognizing nearly every neoepitope presenting a unique mutation. However, certain chemical features may often enriched improve the likelihood of association between a peptide-MHC molecule and a randomly generated TCR. As mentioned previously, protein-protein interfaces are in hydrophobic residues, and the burial of such residues is energetically favorable. Indeed, a recent study of immunodominant viral epitopes highlight an enrichment in hydrophobic residues [43]. Thus, the use of structural knowledge and chemical intuition has applications beyond TCR engineering.

Towards these goals, here we aim to improve our understanding of the relationship between TCR structure and function. By building structural and computational models of large experimental data sets, we can begin to investigate aspects of TCR affinity, specificity, and epitope preferences to facilitate the development of improved immunotherapies.
CHAPTER 2:

MATERIALS AND METHODS

2.1 Common methods applicable to all chapters

2.1.1 DNA mutagenesis, protein expression and purification

Gene constructs for TCR α and β chains were individually inserted into the pGMT7 vector containing an ampicillin resistant gene for selection and an IPTG inducible lac operon. The HLA-A2 and β2-microglobulin (β2m) gene constructs were inserted into the custom pHN1+ vector that has continuous expression requiring no induction. Individual mutations were made by designing complementary primers (Integrated DNA Technologies) and using the Quick-change site-directed mutagenesis kit (Stratagene).

Expression and refolding of soluble constructs of TCRs and HLA-A2 were performed as previously described [44]. Briefly, the TCR α and β chains, the HLA-A2 heavy chain and β2m were generated in Escherichia coli as inclusion bodies, which were isolated and denatured in 8M urea. Peptides were synthesized and purified commercially (AAPTECH) and diluted in DMSO. TCR α and β chains were diluted in TCR refolding buffer (50mM Tris (pH 8), 2mM EDTA, 2.5M urea, 9.6mM cysteamine, 5.5mM cystamine, 0.2mM PMSF) at a 1:1 ratio. HLA-A2 and β2m were diluted in MHC refolding buffer (100mM Tris
(pH 8), 2mM EDTA, 400mM L-arginine, 6.3mM cysteamine, 3.7mM cystamine, 0.2mM PMSF) at a 1:1 ratio in the presence of excess peptide. TCR and pMHC complexes were incubated for 24 h at 4°C. Afterward, complexes were desalted by dialysis at 4°C and room temperature respectively, then purified by anion exchange followed by size-exclusion chromatography (Figure 2.1). Refolded protein absorptions at 280 nm were measured spectroscopically and concentrations determined with appropriate extinction coefficients.

2.1.2 Surface Plasmon Resonance (SPR)

Surface plasmon resonance experiments were performed with a Biacore 3000 or T200 (GE) instrument using CM5 sensor chips as previously described [45]. In all experiments, TCR was immobilized to the sensor chip via standard amine coupling and pMHC complex was injected as analyte. Experiments were performed at 10-25°C in 20mM HEPES (pH 7.4), 150mM NaCl, 0.005% Nonidet P-20. Both kinetic and steady-state experiments were performed with TCRs coupled onto the sensor chip at 50–2000 response units. Injected pMHC spanned a concentration range of 0.1–150 μM at flow rates spanning 5-40 μl/min. Data were processed and fit using a 1:1 binding model utilizing MATLAB 2015b. Figure 2.2 shows a representative sensorogram of a binding event between the DMF5 TCR and HLA-A2/ELAGIGILTV (A) and the resulting equilibrium curve (B). $k_{on}$ values were indirectly calculated from the equilibrium determined $K_d$. 
Figure 2.1 Size exclusion purification of 1G4 TCR. (A) S200 size exclusion chromatogram of the refolded 1G4 TCR. (B) S75 size exclusion chromatogram of 1G4 TCR after heterodimer isolation.
Figure 2.2 Sensorgram of binding between DMF5 and HLA-A2/ELAGIGILTV. (A) The sensorgram shows increasing concentrations of HLA-A2/ELAGIGILTV injections resulting in increasing response. (B) Response Units (RU) measurements taken at equilibrium (black X) are used to generate an equilibrium binding curve. The equations for determining binding kinetics and $K_D$ are based on a monovalent interaction using the law of mass action (A) and the Langmuir binding isotherm (B).
2.1.3 TCR/pMHC Modeling within the Rosetta Protein Design Suite

For structural modeling, Rosetta with the Talaris2013 score function was used (Table 2.1) [46], using the PyRosetta interface [47]. Multiple cycles of backbone minimization and rotamer optimization with heavy atom restraints brought native crystal structures to a local energy minima [48]. Following initial structure minimization, the desired TCR, MHC, or peptide sequence was computationally introduced followed by independent Monte Carlo based simulated annealing trajectories of the TCR CDR loops and/or the peptide. For single point mutations, this was performed using three trajectories of Rosetta’s conservative LoopMover_Refine_CCD mover with 3 outer cycles and 10 inner cycles, using an initial metropolis acceptance criteria of 2.2 that decreased linearly to 0.6 [46]. For thorough structural sampling when modeling novel loops or peptides with limited a priori knowledge, random phi/psi perturbations of the loop/peptide region of interest followed by the more rigorous and computationally expensive LoopMover_Refine_KIC mover generated upwards of 1000 decoy models. The lowest energy structure(s) was retained for further analysis, operating under the assumption that the lowest energy state most accurately represent experimental structures (Figure 2.3). The large number of resulting packing operations in both procedures introduced some minor variability when scoring the models. Therefore, averaging the unweighted score terms for the lowest scoring three trajectories yielded improved consistency for future energy calculations [49].
### Table 2.1

**TALARIS2013 Score Function**

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>fa_atr</td>
<td>Lennard Jones net attractive energy</td>
<td>0.80</td>
</tr>
<tr>
<td>fa_rep</td>
<td>Lennard Jones net repulsive energy</td>
<td>0.44</td>
</tr>
<tr>
<td>fa_sol</td>
<td>Lazaridis-Karplus solvation energies</td>
<td>0.75</td>
</tr>
<tr>
<td>fa_intra_rep</td>
<td>Lennard Jones intraresidue repulsive energy</td>
<td>0.004</td>
</tr>
<tr>
<td>fa_elec</td>
<td>Coulombic electrostatic potential with distance-dependent dielectric</td>
<td>0.70</td>
</tr>
<tr>
<td>pro_close</td>
<td>Proline ring closing energy</td>
<td>1.00</td>
</tr>
<tr>
<td>hbond_sr_bb</td>
<td>Hydrogen bonding energy, short range backbone-backbone</td>
<td>1.17</td>
</tr>
<tr>
<td>hbond_lr_bb</td>
<td>Hydrogen bonding energy, long range backbone-backbone</td>
<td>1.17</td>
</tr>
<tr>
<td>hbond_bb_sc</td>
<td>Hydrogen bonding energy, backbone-sidechain</td>
<td>1.17</td>
</tr>
<tr>
<td>hbond_sc</td>
<td>Hydrogen bonding energy, sidechain-sidechain</td>
<td>1.10</td>
</tr>
<tr>
<td>dslf_fa13</td>
<td>Disulfide geometry potential</td>
<td>1.00</td>
</tr>
<tr>
<td>rama</td>
<td>Ramachandran propensities</td>
<td>0.20</td>
</tr>
<tr>
<td>omega</td>
<td>Omega dihedral</td>
<td>0.50</td>
</tr>
<tr>
<td>fa_dun</td>
<td>Internal energy of sidechain rotamers as derived from Dunbrack's statistics</td>
<td>0.56</td>
</tr>
<tr>
<td>p_aa_pp</td>
<td>Probability of amino acid at ( \Phi/\Psi )</td>
<td>0.32</td>
</tr>
<tr>
<td>ref(^1)</td>
<td>Reference energy for each amino acid.</td>
<td>1.00</td>
</tr>
</tbody>
</table>

1. The parameters for each amino acid used by the ref energy term are as follows:

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>0.592942</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.354993</td>
</tr>
<tr>
<td>Aspartate</td>
<td>-1.28682</td>
</tr>
<tr>
<td>Glutamate</td>
<td>-1.55374</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.43057</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.140526</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.357498</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.831803</td>
</tr>
<tr>
<td>Lysine</td>
<td>-0.287374</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.602328</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.158677</td>
</tr>
<tr>
<td>Asparagine</td>
<td>-0.94198</td>
</tr>
<tr>
<td>Proline</td>
<td>-0.219285</td>
</tr>
<tr>
<td>Glutamine</td>
<td>-1.17797</td>
</tr>
<tr>
<td>Arginine</td>
<td>-0.14916</td>
</tr>
<tr>
<td>Serine</td>
<td>0.176583</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.16454</td>
</tr>
<tr>
<td>Valine</td>
<td>0.744844</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.92933</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.131696</td>
</tr>
</tbody>
</table>
Figure 2.3 Representative energy funnel of peptide conformational states. Randomly sampled conformations are associated with varying degrees of energy. Given enough sampling, the lowest energy conformation most likely represents the native structure.
2.2 Methods for Chapter 3

2.2.1 Score Function Training

To develop a new score function for predicting changes in binding $\Delta \Delta G^*$, we considered Rosetta full atom terms in addition to dynamically derived terms (bound and free order parameters and RMS fluctuations). Multiple linear regression was performed in MATLAB 2015b using measured $\Delta \Delta G^*$ values. A stepwise elimination protocol was used to remove contextually insignificant terms. A k-fold ($k = 10$) cross validation was performed with the data points and significant predictor terms [50].

2.2.2 Modeling Explicit Water and Non-Standard Amino Acids

To model and score buried water molecules and the non-standard amino acids, explicit TIP3P waters and non-standard amino acid parameters were enabled in Rosetta. Water molecules were placed at their initial crystallographic coordinates followed by 100 high-resolution docking trials to coordinate the water molecule in the pocket of the interfaces. The water coordinates were then fixed in position relative to the pMHC for TCR point mutation modeling.

2.2.3 Molecular Dynamics Simulations

Molecular dynamics simulations were calculated utilizing the AMBER molecular dynamics suite as previously described [51]. Results for the free and bound A6 and DMF5
were taken from these simulations, with other simulations following the same protocol. Briefly, coordinates for the complexes with the LC13, B7, 1G4, Mel5, RD1 and DMF4 TCRs were obtained from PDB accession codes 1MI5, 1BD2, 2BNR, 3HG1, 5E9D, and 3QDM. Coordinates for the free TCRs were obtained by stripping away the pMHC. Prior to simulation, starting systems were charge neutralized with explicit Na+ counterions and solvated with explicit SPC/E water. Following this, systems were energy minimized and heated to 300 K with solute restraints. Afterwards, solute restraints were gradually relaxed and followed with 2 ns of simulation with no solute restraints for equilibration, after which 100 ns production trajectories for all systems were calculated. Trajectories were calculated using GPU-accelerated code. Trajectory analysis including calculation of RMSF values used the ccptraj from the AMBER suite [52]. Order parameters were calculated using isotropic reorientational eigenmode dynamic analysis using vectors defined from the Cα to Cβ (or Cα to H for glycine) atoms. For double mutants, descriptors were averaged between the two positions for scoring purposes (i.e. for mutant XY, the RMSF of position X is averaged with the position Y RMSF to give an RMSF descriptor for XY).

2.3 Methods for Chapter 4

2.3.1 Clustering and Peptide Selection

Using yeast display, Garcia and colleagues screened peptide libraries encoded by genes for a single chain peptide/HLA-A2. Staining this library with the DMF5 TCR identified
1452 unique peptides distributed across over 300,000 yeast clones. 95% of the clones encoded 1 of the 10 most frequently observed peptides. Clustering the 1452 unique sequences by Levenshtein distance (sequence similarity) identified 10 sequentially distinct peptide clades, centered on one of the aforementioned 10 peptides.

2.3.2 Differential Scanning Flourimetry on pep/MHC Complexes

Thermal stability for each peptide/HLA-A2 was measured with differential scanning flourimetry, with SYPRO Orange to detect changes in hydrophobicity during denaturation [53]. Briefly, 10-20 μM pMHC buffered in 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, with 0.005% surfactant P20 was incubated with excess SYPRO Orange for a total solution volume of 20μL. For stability measurement, the scan rate was fixed at 1°C/minute, and fluorescence intensity recorded at 607 nm. The temperature range spanned from 25°C to 95°C, and the midpoint between peak fluorescent intensity at which the transition was 50% complete was taken as apparent Tm.

2.3.3 Crystallization and Data Collection

Crystals of the DMF5 SMLGI GIVPV/HLA-A2 complexes were grown from 13% PEG 3350, 0.25M MgCl₂ buffered with 0.1 M Tris (pH 8.1) at 25°C. Crystals of the DMF5 MMWDRGLGM M/HLA-A2 complexes were grown from 18% PEG 3350, 0.25M MgCl₂ buffered with 0.1 M HEPES (pH 7.9) at 25°C. Crystals of the free MMWDRGLGM M/HLA-A2
complexes were grown from 25% PEG 1000, 0.01% NaN3 buffered with 0.1 M MES (pH 6.5) at 25°C. Crystallization was performed using hanging drop/vapor diffusion. For cryoprotection, crystals were transferred into 20% glycerol/80% mother liquor for 30 s and immediately frozen in liquid nitrogen. Diffraction data were collected at the 22ID (SER-CAT) beamline at the Advanced Photon Source, Argonne National Laboratories. Data reduction was performed with HKL2000. The ternary complexes were solved by molecular replacement using PHENIX and Protein Data Bank (PDB) entry 3QDG as the reference model [54]. Rigid body refinement, followed by multiple steps of restrained refinement were performed with phenix.refine [55]. Atomic positioning was verified with an iterative-build OMIT map calculated in PHENIX. Evaluation of models and fitting to maps were performed using COOT [56]. Structures were visualized using PyMOL[57].

2.3.4 Epitope Selection and Design

For simplicity, residue selection was limited to those observed in the yeast libraries (Figure 2.3). Residue combinations were modeled and scored as described, with 3QDG serving as the template for GXGX based peptides (Figure 2.4A) and the DMF5/MMWDRGLGMM/HLA-A2 structure serving as the template for DRG peptides (Figure 2.3B). The lowest scoring epitope combinations were selected for experimental analysis.
Figure 2.4 **Combinatorial residue selection for epitope design.** (A) Residues available to model epitopes in GXGX conformation for a total of 6048 possible epitopes. (B) Residues available to model epitopes in DRG conformation for a total of 12288 epitopes.
2.4 Methods for Chapter 5

2.4.1 Epitope collection for molecular modeling

We used four independent sets of previously published data to access the validity of correlating epitope structural features to immunogenicity. The first data set consisted of 11 wild-type/neoeptope pairs sequenced from BALB/c mice, published by Duan et al [42]. Five of the 11 neoepitopes resulted in tumor rejection when used to immunize BALB/c mice [42], and we used this binary criterion to gauge energetic predictive performance qualitatively. We refer to this data set as the Srivastiva data set.

With the next data set, the authors recorded the survival rates of 110 melanoma patients treated with the checkpoint-blockade inhibitor, ipilimumab [58]. Whole exome sequencing determined the mutational load of each patient, along with 14,028 HLA-A2 associated neoepitope/wild-type pairs. We used this information to guide our high throughput structural modeling and confirm observations observed with the Srivastiva data set. We refer to this data set as the Garroway dataset.

We built the neural network training data set from multiple published sources using proteomics and quantifiable, in vitro data. To build a generalizable model to predict structural features of immunogenicity, we considered three ‘types’ of peptides: MHC incompatible peptides, ‘self’ (not immunogenic) peptides, and immunogenic peptides. For simplicity, we only considered nonameric peptides. 1044 HLA-A2 incompatible peptides were selected from netMHC training sets [59], with a measured affinity of >50,000nM. For self, non-immunogenic peptides, proteomics revealed 2757 human
peptides bound to HLA-A201 when eluted from HeLa cells [3, 60]. These peptides were partially confirmed to not initiate an immune response. Finally, we screened the IEDB for HLA-A201 specific peptide sequences with reported ELISPOTS greater than 50%, yielding 155 immunogenic epitopes. Thus, the total training data set consisted of 3955 HLA-A201 associated peptides and is henceforth referred to as the Skynet data set.

The final data set was isolated from the Skynet data set and used to test the neural network performance after training and cross validation. The authors of this data set reported extensively on the MHC stability, binding affinity, and immunogenicity of 13 nonameric variants of the tumor antigen NY-ESO. We refer to this data set as the Luescher data set.

2.4.2 Epitope structural modeling

The Srivastiva data set consists of 11 wild-type/neoeptiophe pairs in context of the mouse MHC class I molecule, H-2K\textsuperscript{d}. This particular MHC allele is not well studied, with few crystal structures in the PDB database [61, 62]. The length of the neoepitopes ranged from 8-11 residues, which presented a unique modeling challenge as the best-characterized structures only present a viral peptide nine residues long [62]. To account for this, the peptide of the H-2K\textsuperscript{d} PDB template, 2FWO [62], was adjusted to the desired length and computationally mutated to the desired sequence. The peptide anchors were then fixed in place and the backbone of the peptide allowed to energetically minimize in context of the H-2K\textsuperscript{d} molecule, along with sidechain repacking. To account for the
increased degrees of conformational plasticity, 200 independent trajectories for each peptide pair were generated. Each structure was ranked by the Talaris2013 score function and the top 10 structures selected for further evaluation. Of the top 10 structures, final neoepitope conformations were selected based on structural similarity to the predicted wild type peptide model.

Modeling the epitopes of the other data sets was more generic. For modeling simplicity and clarity, only nonamers were considered. The pMHC structure 3QFD served as a template, and at least 10 decoys for each epitope was energy minimized with the Talaris2014 score function. The energy scores of the three lowest scoring structures were averaged together and used for further analysis.

2.4.3 Neural network training

The neural network training is performed in a manner similar to that described by Nielson et al [41]. The test and training of the neural networks is performed using a fivefold cross-validation by splitting the 3955 peptides from the Skynet dataset into five sets of training, validation, and test data. The splitting is performed such that all sets have approximately the same distribution of weak binding, self, and immunogenic peptides. With a classification criteria of immunogenic or not immunogenic, the training data are used to perform feed-forward and back propagation, the validation set is used to define the stopping criteria for the network training, and the test set is used to evaluate performance via AUC. All data sets are rotated to ensure every set is used in training,
validation, and testing and the average AUC of the test sets is reported as an indicator of overall performance. To maintain an equal distribution of classifiers and eliminate bias for not-immunogenic peptides, immunogenic peptides in the training sets were randomly oversampled.

The neural network architecture used is a conventional feed-forward network [63] with an input layer with 80-117 neurons, one hidden layer with 1–10 neurons, and a single neuron output layer. The neurons in the input layer encode the 9 amino acids in the peptide sequence with each amino acid represented by up to 11 neurons describing structural features from the structural models. The remaining 18 neurons describe global structural features of the entire pMHC model. For each of the five training and test sets, a series of network trainings were performed each with a different number of hidden neurons (2, 3, 4, 6, 8, and 10) and a different number input neurons. For each series, a single network with the highest test performance was finally selected (Figure 2.5).
Figure 2.5 **Neural network cross validation training results.** A cross-validation procedure interrogating a network with 81 input neurons demonstrates optimal performance is with 5 hidden neurons. Performance decreases with more neurons, indicating the data is overfit.
CHAPTER 3:

A GENERALIZED FRAMEWORK FOR COMPUTATIONAL DESIGN AND MUTATIONAL SCANNING OF T CELL RECEPTOR BINDING INTERFACES

3.1 Introduction

This chapter discusses findings from recently published articles [64, 65], which describe the training and application of a protein design modeling procedure to predict the effects a given TCR mutation will have on binding a target peptide/MHC ligand.

Recent advances have highlighted the potential therapeutic uses for TCRs with altered binding properties. As T-cell potency can be improved with antigen affinity [66], clinical trials with gene-modified T cells have explored the use of engineered, high affinity TCRs for improved antigen targeting [36]. High affinity TCRs are also used as the antigen recognition component of soluble reagents designed to redirect naive, unmodified T cells [67].

Multiple studies have aimed to generate high affinity TCRs in order to enhance sensitivity and selectivity. Recent outcomes however have suggested that fine manipulation of TCR binding, with an emphasis on specificity, may be more valuable than large affinity increments [68]. Hypothetically, if the addition of TCR ‘glue’ is directed more
towards the MHC protein than the peptide, the likelihood of off-target recognition is increased. Additionally, there is a poor understanding of the relationship between TCR affinity and potency. Although some very high affinity TCRs show considerable sensitivity [69], in other cases improving affinity outside an optimal window or above a threshold has led to decreased potency [69].

As mentioned earlier, computational structure-guided design is ideally suited for improving a TCR binding interface, and the Rosetta Protein Design Suite popularized the concept of stochastic structural perturbations in combination with score functions to rapidly score and select structures for optimal energetics [47, 70]. Although the use of in vitro evolution has led to the majority of high affinity TCRs, structure-guided computational design offers the potential for finer control over TCR affinity and specificity. These approaches can manipulate interactions in a way that more appropriately address peptide specificity, with tightly controlled affinity increments.

Here we studied the generality of TCR structure guided design. This work found that previous approaches, which successfully optimized the binding of a therapeutic TCR, had lower accuracy when applied to a broader set of TCR interfaces. Thus, we sought to develop a more general-purpose methodology for TCR design. Using a large dataset of experimental binding measurements spanning multiple TCR interfaces, a new scoring function that explicitly accounted for unique features of each interface, most notably flexibility, was trained. Together with other improvements, this function allowed for design of affinity-enhancing mutations in multiple TCRs, including those not used throughout the training process. This new methodology also captured the impacts of
mutations and substitutions in the peptide/MHC ligand. In light of the observation where T cell potency is associated with TCR affinity [69, 71] and other factors [72], this approach offers a generalizable and efficient method to design affinity-enhanced TCRs for therapeutic use.

Additionally, we compared sequence fitness landscapes derived from our structure guided design approach and a traditional directed evolution experiment. Sequence fitness landscapes offer a powerful perspective of protein-protein interactions not available from structural data alone by experimentally determining, on a residue-by-residue basis, which amino acids contribute to binding as well as the most optimal amino acids at each position [73]. By combining directed evolution with deep sequencing, it is now possible to generate sequence fitness landscapes that survey the impact of every amino acid substitution across the entire protein-protein interface. More recently, Kranz and colleagues reported the use of single-codon libraries with the RD1-MART1\textsuperscript{HIGH} TCR to generate sequence fitness landscapes that allowed analysis of the impact of each residue on binding to a specific peptide/HLA-A2 complex [73]. As an immediate comparison with our in-silico approach to evaluating these interactions, we found our computational model also identified some of the most enriched residues identified in the RD1-MART1\textsuperscript{HIGH} sequence fitness landscape. However, our model also identified mutations that did not yield substantial enrichment in the yeast libraries. This is likely due in part to the sensitivity of yeast surface display to not only changes in binding, but also changes in protein stability. The \textit{in silico} approach nonetheless performed well when focused on mutations experimentally shown to improve binding and proved advantageous for
providing structural interpretations. Overall, we highlight the complementary strengths of computational modeling and yeast surface display coupled with deep mutational scanning for engineering therapeutic TCRs.

3.2 Results

3.2.1 Application of earlier structure guided design methods to a large data set

Based on previous work with the A6 TCR [74], our lab recently described a modeling and scoring scheme to predict the structural and energetic effects of point mutations within interfaces of the αβ TCR, DMF5 [75]. This approach identified several affinity enhancing mutations in DMF5 which when combined led to affinity enhancements towards peptide/class I MHC molecule HLA-A2 of up to 400-fold. To explore the generality of this approach, similar methodology was applied to 94 independent ΔΔG° values resulting from single amino acid mutations collected from four TCR-pMHC interfaces using both previously published and new data [45, 74, 75]. The limitations of the previous approach [75] became apparent as correlations with this larger data set yielded a Pearson’s coefficient of only 0.16 (Figure 3A). Thus, in light of the low correlations between prediction and experiment, we identified the need for a more generalizable framework for modeling and predicting point mutations across multiple TCR-pMHC interfaces.
Figure 3.1 Training a modeling and scoring scheme with a large collection of TCR mutation $\Delta \Delta G^\circ$ data. (A) Application of method described in Pierce et al. performs poorly with a larger data set. (B) Training the Rosetta specific terms improves correlation, but performance is still weak. (C) Replacing binding score with complex scores further improves predictions. (D) Incorporating RMSF features gives the final TR3 modeling and scoring scheme. The best fit line and correlation coefficient is indicated for all plots. The 95% confidence interval is shown for initial and final plots A and D. An off-scale prediction score of 26 (DMF5 $\alpha$G28L) is denoted by a black arrow.
3.2.2 Development of a generalized TCR-pMHC scoring function

To develop a generalizable TCR scoring function, we considered 16 full-atom Rosetta terms commonly used for protein design and structure prediction [46]. Using the Rosetta terms as predictor variables and experimental binding energies of the dataset described above as the response variable, we used multi-linear regression to parameterize a starting score function for estimating the effect of the various point mutations on $\Delta \Delta G^\circ$. The most significant contributors to the model (P < 0.05) described van der Waals attractive forces and solvation effects. However, the correlation between binding score and $\Delta \Delta G^\circ$ remained low ($R = 0.43$; Figure 3B). Thus, we did not explore removing insignificant features at this stage in favor of obtaining a more robust prediction model.

Ideally, binding energy calculations would utilize structural information for both the free and bound molecules [76]. However, structures of free TCRs and pMHCs can vary between free and bound states [51], and the large surface areas of receptor and ligand binding sites possess significant conformational degrees of freedom. We thus focused only on relative effects by scoring only TCR–pMHC complexes, rather than scoring the complex and the two free proteins as described above. We refer to the difference in scores between wild type and mutant complexes as ‘complex scores’. This approach comes with a limitation in that complex scores do not account for energies in the free TCR associated with making the mutation (i.e. the $\Delta G^\circ$ for TCR WT $\leftrightarrow$ TCR mutant). Ideally, the subtraction of these energies are required when examining the impact of a mutation on binding. There are two potentially significant consequences to this. First, an improved
complex score could arise solely due to improved interactions within the TCR (i.e. better
TCR stability). We minimized the impact of this by focusing on sites that are in proximity
to the ligand and thus more likely to influence binding. Second, this approach ignores any
effects on binding if they stem from conformational changes in the free TCR.

Using the same 16 full-atom Rosetta terms, a multi-linear regression of complex
scores vs. $\Delta \Delta G^*$ yielded an improved function ($R = 0.66$ for complex scores, vs. $R = 0.43$
for binding scores; Figure 3C). Despite the theoretical limitations noted above, complex
scores are therefore more applicable for our framework and thus used for all further
calculations. The improvement using complex scores may reveal underlying limitations in
the energy function terms and/or limitations in recapitulating conformational differences
between free and bound TCRs as noted above, leading to inaccuracies when ‘binding
scores’ are computed. The inherently weak affinities and correspondingly poor quality of
TCR–pMHC interfaces (compared, e.g. to high affinity antibody-antigen interfaces) could
also contribute to why complex scores outperform binding scores.

3.2.3 Refinement of the regression model to include flexibility

Although utilization of complex scores improved the correlation between
prediction and experiment, we sought to identify additional predictors of TCR binding
affinity that might further improve performance. One of the differences between TCRs is
their degree of binding loop flexibility, particularly for the hypervariable CDR3$\alpha$ and
CDR3$\beta$ loops [51]. Although various methods for conformational sampling such as
stochastic loop perturbations or generation of structural ensembles exist [77], these are computationally expensive. To more simply address the impacts of TCR loop flexibility, we considered descriptors from molecular dynamics (MD) simulations of the free and bound TCRs. We recently described a comprehensive MD study of the free and bound A6 and DMF5 TCRs [51] using an experimentally benchmarked simulation methodology. We performed similar simulations on the free and bound B7 TCR. From these simulations root mean square (RMS) fluctuations for each α carbon were determined along with Cα-Cβ (Cα-H for glycine) and Cα-C order parameters to quantify nanosecond-timescale backbone motions. Due to the time that would be required to simulate dozens or hundreds of mutations, we simulated only the wild-type TCRs and their complexes. We then treated fluctuation values and order parameters as ‘positional modifiers’ for each amino acid position, biasing positions for design based on their relative flexibility in the wild-type free and bound structures. Although necessary for throughput, this approach makes the limiting assumption that any given mutation does not influence backbone flexibility on the nanosecond timescale.

To determine if inclusion of RMS fluctuations and/or order parameters could lead to an improved scoring function, we included these six terms along with the 16 full-atom Rosetta terms in a multilinear regression of complex scores vs. ΔΔG°, coupled with a stepwise elimination protocol [50]. This fit identified six significant (P < 0.05) features: four structural terms (van der Waals attractive and repulsive forces, solvation energies and sidechain hydrogen bonding) and two flexibility terms (RMS fluctuations for α carbons of the free and bound structures). A structural term weighting Ramachandran
angle propensities was borderline significant (P = 0.11), but was retained to help identify and exclude structural models with residues forced into unrealistic conformations.

The regression models estimated the weights of the RMS fluctuation features to be negative, suggesting flexible positions are more favorable to target for design (although mobility in the complex was weighted more heavily as discussed below). For a critical examination of the significance of this determination, models with and without the fluctuation terms in addition to the five Rosetta terms were generated and compared. Akaike information criterion (AIC) [78] found the incorporation of features describing flexibility resulted in a 99.8% likelihood of a superior prediction model. Bayesian information criterion (BIC) [78] more strongly penalized additional terms, yet also indicated that inclusion of the fluctuation terms improved the regression model beyond random chance.

Finally, a k-fold cross validation (k = 10) [50] was used to validate and estimate overall predictive performance. From this analysis, the RMS error (reflecting the difference between experimental and predicted ΔΔG° values) was estimated as 0.81 kcal/mol, with an impressive correlation of 0.71 (Figure 3D; note this correlation includes accounting for structural water as described below). For comparison, our previous approach with the Rosetta interface score function yielded a correlation of only 0.16 (Figure 4B), and a recent analysis of protein design approaches estimated an average error of 1.2 kcal/mol for protein–protein interactions [79]. Table 1 shows the terms and weights for the final regression model, termed the TR3 score function.
TABLE 3.1
TR3 SCORE FUNCTION

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
<th>Weight</th>
<th>Error$^a$</th>
<th>p-value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td></td>
<td>2.29</td>
<td>0.35</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fa_atr</td>
<td>van der Waals net attractive energy</td>
<td>0.21</td>
<td>0.03</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fa_rep</td>
<td>van der Waals net repulsive energy</td>
<td>0.05</td>
<td>0.01</td>
<td>0.005</td>
</tr>
<tr>
<td>Fa_sol</td>
<td>Solvation energies</td>
<td>0.18</td>
<td>0.08</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hbond_sc</td>
<td>Sidechain-sidechain hydrogen bonding energies</td>
<td>0.34</td>
<td>0.09</td>
<td>0.008</td>
</tr>
<tr>
<td>Rama</td>
<td>Ramachandran propensities</td>
<td>0.12</td>
<td>0.05</td>
<td>0.119</td>
</tr>
<tr>
<td>RMSF_bound</td>
<td>Bound RMS Fluctuation values</td>
<td>-0.82</td>
<td>0.30</td>
<td>0.049</td>
</tr>
<tr>
<td>RMSF_free</td>
<td>Free RMS Fluctuation values</td>
<td>-0.36</td>
<td>0.10</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Estimated total error: 0.81 kcal/mol$^c$

$^a$Determined as 1.96 standard deviations of k-fold cross validation weights

$^b$p-value for the F statistic of the hypotheses test that the corresponding coefficient is equal to zero

$^c$Average test RMS error from k-fold cross validation
3.2.4 Accounting for energetically significant structural water improves predictions

Rosetta utilizes an implicit solvation model to estimate solvation energies associated with bulk water [80]. However, TCR–pMHC interfaces are large and buried water molecules are often observed crystallographically. In some instances, these structural waters play key roles in the interface that would not be captured with an implicit solvation model [81]. Indeed, many predicted mutations that filled the void of an interfacial water molecule in the interface with the DMF5 TCR resulted in a falsely favorable score. For example, Ser99 in the DMF5 β chain engages the peptide, but is also involved in a complex water-mediated hydrogen bond network linking the peptide to the TCR (Figure 3.2A). The predicted impacts of mutations at this position did not correlate well with experiment (Figure 3.2B), consistent with a determination that this water molecule is structurally and energetically significant. Accounting for the impact of this molecule, we docked the buried water in the DMF5 interface into its corresponding pocket and treated it explicitly in modeling and scoring. This improved the agreement between prediction and experiment for Ser99β point mutations without altering the predictions for distant residues (Figure 3.2C). Further design efforts incorporated this technique when we detected crystallographic water molecules buried in the interface between peptide and TCR.
Figure 3.2 Accounting for buried structural water improves predictions. (A) A buried water molecule observed crystallographically in the DMF5-MART126{27L}-35/HLA-A2 interface forms multiple electrostatic interactions between the TCR and peptide. (B) The correlation between prediction and experiment for models of DMF5 point mutants scored with TR3 is 0.63 when the buried water molecule is ignored. Five mutations at position 99β are indicated and are responsible for the low correlations. (C) The correlation between prediction and experiment for DMF5 point mutants improves to 0.80 when the buried water molecule is treated explicitly.
3.2.5 Overall performance including data not used for training

We next evaluated the overall performance of the modeling and scoring procedure by comparing predicted and measured impacts of TCR mutations in five different interfaces, mutations to HLA-A2, and peptide substitutions not used in training. Previously published and unpublished double mutants in the DMF5, B7, and A6 TCRs were also included [74, 75]. Altogether, performance was excellent, with strong agreement between predicted and experimental impacts on binding over a range of approximately seven kcal/mol in binding free energy (Figure 3.3). Thus the new framework is applicable not only to TCRs, but to mutations in the class I MHC protein HLA-A2 and peptide variants as well, further demonstrating its utility as a generalizable approach for modeling and predicting variations in TCR-pMHC interfaces.

3.2.6 Evaluating sequence fitness landscapes experimentally and computationally

In order to understand the most expeditious strategy to generate higher affinity binding leads from a protein, we explored two approaches with the RD1-MART1\textsuperscript{HIGH} TCR. The first approach suggested substitutions from single-codon libraries that yielded increases in the number of yeast clones expressing antigen-selected TCR, characterized as enrichment values (Figure 3.4A). In the second, the results of these experimental approaches were compared to our fully trained \textit{in silico} computational approach. Here we used the structure of the RD1-MART1\textsuperscript{HIGH: MART1\textsubscript{26(27L)-35}/HLA-A2 complex to predict
Figure 3.3 All point mutation data examined in training, developing, and evaluating the TR3 score function. The overall correlation between prediction and experiment is 0.79 when including point mutation binding data from unrelated TCRs, HLA molecules, and peptides.
Figure 3.4 **Sequence fitness landscapes of the RD1-MART1<sup>HIGH</sup> TCR.** (A) Enrichment of single amino acid substitutions in antigen (HLA-A2/MART1)-selected yeast clones expressing the RD1-MART1<sup>HIGH</sup> TCR. (B) A high enrichment value is indicative of a favorable substitution. TR3 energy scores based on the PDB coordinates 5E9D. A lower energy score indicates a predicted increase in affinity for HLA-A2/MART1<sub>26(27L)-35</sub>. 
the structural consequences and the energetic impacts of every individual substitution in
the CDRs (Figure 3.4B), and used this information to interpret the deep sequencing data.

Based on the sequence fitness landscape of RD1-MART1\textsuperscript{HIGH} performed by Kranz
and colleagues, 8.3% of the mutations showed enrichment above the wild-type TCR. We
next evaluated the same residues from the sequence fitness landscape computationally,
examining all 20 amino acids in the 39 CDR loop positions explored in the RD1-MART1\textsuperscript{HIGH}
TCR (Note: values less than 1 considered candidates for improved affinity). There was a
general, qualitative agreement between prediction and experiment, as the majority (92%)
of the mutations predicted to weaken affinity were also poorly represented in the yeast
libraries (Figure 3.5A). However, 25% of the negatively enriched mutations were also
predicted to improve binding. The majority (78%) of the negatively enriched mutations
predicted to improve binding were mutations that reduced polarity, with 62% involving
introduction of a hydrophobic amino acid. This suggests that some of the discrepancy
between computational prediction and sequence enrichment may be due to changes in
protein stability. This is a well-characterized selection parameter in yeast display [1], but
is not an output of a computational design approach focused solely on improving affinity.
The mutation of A99β is a good example. Mutation of A99β to Tyr improved affinity over
4.5 fold [65]. From our molecular models, we predicted the mutation of A99β to Trp to
have an even more substantial impact on affinity as the modeling indicated the Trp side
chain would pack more tightly against the peptide. However, modeling A99β with Trp also
resulted in the added exposure of ~50 Å\textsuperscript{2} of hydrophobic surface area (compared to
substitution with Tyr). Using well-recognized estimates of 20-30 kcal/mol in free energy
Figure 3.5 Comparison of prediction score and enrichment ratio. (A) Plots of the TR3 prediction score and enrichment ratio show qualitative agreement. The horizontal dashed line indicates a binding score of 0.92, above which binding is predicted to be weaker compared to wild type. The four most enriched mutants are shown in red, and four favorably predicted mutants shown in green. (B) Prediction score vs. enrichment ration with each data point colored by the predicted change in hydrophobic solvent accessible surface area.
per Å² of exposed hydrophobic surface area [82], the resultant destabilization could be as high as ~1.5 kcal/mol.

A wider comparison of enrichment vs. predicted changes in hydrophobic solvent accessible surface further suggested that sequence enrichment in the fitness landscapes was at least in part associated with reduced exposed hydrophobic surface area, and vice versa (Figure 3.5B). Of the residues with log2 enrichment ratios ≥1, we predict 68% to have reduced solvent exposed hydrophobic surface. In contrast, for those residues with enrichment ratios ≤1, we predict 66% to have increases in exposed hydrophobic surface.

To test if reduced yeast surface levels of the properly folded mutants were in part responsible for lower enhancement values, our collaborators examined four individual mutants. We predicted these mutants to have higher affinities, yet they were observed to have negative enrichment values (Figure 3.5A). The four mutants (A99Wβ, S94Mα, G28Wα, G28Fα) were compared to the wild-type RD-MART1HIGH TCR for binding to MART126(27L)-35/HLAA2 monomers using flow cytometry. Consistent with the notion that negative enrichment values could be due to lower surface levels of the properly folded proteins, all four mutants, and especially the S94Mα, G28Wα, and G28Fα mutants, were expressed at significantly lower surface levels than wild-type RD1-MART1HIGH TCR. Although the A99Wβ mutant appeared to have a similar affinity to the wild-type RD-MART1HIGH TCR, based on the monomer titrations, it was not possible to estimate affinities for the other three mutants due to their low surface levels.
Building upon the previous observations, Harris et al. selected eight mutations with enrichment values ranging from 10- to 51-fold (aT91Q, bA99Y, aG28E, aS61G, aY50W, bN28G, aK97Q) for individual expression on the surface of yeast and binding assays to the MART1_{26(27L)-35}/HLA-A2 ligand. If we restrict our focus solely to these mutations with binding measurements, after computing binding ΔΔG° relative to RD1-MART1^{HIGH} using the reported values [65], moderate agreement between computation and experiment is observed, with a correlation coefficient of 0.68 (Figure 3.6A). This analysis however includes the N28Gβ and K97Qα mutations. Neither of these positions interacts directly with peptide/MHC either in the wild type complex or in the models of the mutants through either short- or long-range interactions (the closest predicted approaches for the N28Gβ and K97Qα mutants are 16 and 11 Å, respectively). Excluding these mutants from the comparison substantially improved the correlation between prediction and experiment (R = 0.91). Prediction scores of the double and triple mutants also corroborated well with experiment, with a correlation coefficient of 0.87 when these were included in the comparison (Figure 3.6B). The correlations are consistent with the training and validation of our in silico method, further demonstrating the utility of our computational method in real-world applications.
Figure 3.6 *Prediction scores versus measured ΔΔG for each of the experimentally studied RD1-MART1^{HIGH} mutants.* (A) When including all single, double and triple mutants examined, the correlation coefficient (R) was 0.68. (B) As in panel A, but with the two mutants that are more distant (N28Gβ and K97Qα) excluded from the comparison; the correlation coefficient (R) was 0.87.
3.3 Discussion

There is significant interest in enhancing TCR affinity to improve antigen sensitivity, and accordingly, numerous high affinity TCRs have been generated. Although T-cell potency has been shown to improve with affinity, questions remain about the existence of optimal affinity windows or thresholds and the merits of large vs. incremental improvements in binding affinity [19]. Additionally, following adverse events in clinical trials [34], there is a growing recognition of the importance of evaluating and controlling specificity in affinity-enhanced or otherwise modified TCRs.

In principle, structure-guided computational design offers the potential for fine manipulation of TCR binding properties. Structure-guided design has been used to generate a small number of high affinity TCRs, as well as manipulate binding specificity [75]. However, although the TCR–pMHC structural database has grown significantly in recent years, wide-scale application of structure-guided TCR design is hindered by several complexities. These include the complex architecture of the TCR–pMHC interface as well as the varying degrees of diversity and molecular flexibility in both receptor and ligand [51].

Our improved framework for TCR design permitted the identification of new affinity-enhancing mutations in multiple interfaces. In addition to the expected structural terms descriptive of TCR binding energetics (van der Waals attractive and repulsive forces, solvation energies, and sidechain hydrogen bonding) [76], the regression models estimated the weights of the RMS fluctuation features to be negative, suggesting flexible
positions are more favorable to target for design. Accounting for flexibility is an important aspect of our improved framework, as varying degrees of CDR loop, MHC and peptide flexibility is a characteristic feature of TCRs and pMHC complexes [83]. As with other efforts in protein design, we relied on MD simulations to incorporate flexibility. However, as opposed to simulating structures to identify alternate configurations or generate structural ensembles [84], we added ‘positional modifiers’ that report on amino-acid level motional properties as terms in the score function. We chose this approach as it greatly simplifies the treatment of flexibility, requiring only single MD trajectories for the free wild-type TCR and the TCR–pMHC complex. Of the properties considered, Cα RMS fluctuations were most significant and thus were incorporated into the final function. The weights for these terms were negative, indicating that positions that are more flexible are more favorable for design. There is some anecdotal evidence to support this: in the A6 TCR, the hypervariable CDR3β loop is by far the most mobile, and multiple mutations within this loop improve A6 binding [85]. This could reflect a form of the ‘fly-casting’ effect, in which mobile sites in a receptor are more adept at finding compatible partner sites in a ligand. Interestingly, the flexibility weights were larger for residues in the bound state. While residual mobility in TCR–pMHC interfaces has been observed and this term could be accounting for this [51], it is also possible that in the complex this term helps overcome limited conformational sampling in modeling.

Additionally, our computational design approach for engineering TCRs provided an opportunity to evaluate the sequence fitness landscapes computationally and compare enrichment with predicted impacts on binding. While there was qualitative
agreement between those mutations predicted to weaken binding and sequence depletion, the \textit{in silico} approach did not distinguish well between mutations predicted to improve binding and sequence enrichment, as 25\% of mutations selected against were predicted to enhance binding. Much of this may reflect the sensitivity of yeast surface display to changes in protein stability, resulting in some substitutions that are either reduced or enhanced in surface levels. Support for this hypothesis is in our analysis of the modeled structures of each mutant for changes in exposed hydrophobic surface area: sequences enriched showed a much stronger trend to reduce hydrophobic solvent accessible surface area, and vice versa. There can be a tendency for structure-guided design to select for hydrophobic mutations \cite{86} and our findings reiterate that improvements in \textit{in silico} approaches may be found from continued attention to electrostatic features such as polar solvation, hydrogen bonding, and interactions with formal charges \cite{76}. However, for those mutants studied directly, our \textit{in silico} analysis showed very good agreement between predicted and impacted effects on binding, permitting the use of the structural models in helping to assess how the mutations acted to improve binding.

Fundamentally, the work described above presents a detailed analysis of variations in TCR-pMHC interfaces, accounting for flexibility and specific solvent considerations through a computationally inexpensive approach. This analysis led to the identification of additional high affinity mutations, demonstrating a broad understanding of the energetics driving TCR/pMHC interactions. Although the affinity gains were
modest, mutations may be combined rationally to offer fine control and obtain target affinities for maximum cellular response.
CHAPTER 4: TCR CROSS REACTIVITY CAN MANIFEST THROUGH UNFORESEEN STRUCTURAL AND SEQUENTIAL MECHANISMS

4.1 Introduction

This chapter discusses the unique structural requirements of TCR specificity and the many mechanisms TCRs may utilize to engage a diverse set of peptides. We are currently organizing these findings for publication.

While specificity may be a hallmark of the immune system, TCRs themselves recognize multiple pMHC complexes[87]. Indeed, the combinations of unique antigens is orders of magnitude larger than the number of existing TCRs in an individual [32, 33]. However, the true extent of TCR specificity and cross reactivity remains largely a speculative issue. Experimentally, a single TCR has been shown to recognize more than a million peptides [38], presenting an obstacle to cell-based therapeutics. Indeed, a recent clinical trial underscored the need for improved methods to define TCR specificity after melanoma patients treated with TCR-engineered T cells suffered from fatal cardiovascular toxicity arising from the unpredicted recognition of a muscle-specific peptide [34, 36].
Although a target size in the millions comprises a considerable number of potential ligands, the makeup of such target pools will not be random but governed by the structural and physicochemical properties of a given TCR’s binding site [39]. Work demonstrating a tendency for TCRs to focus on select regions, or “hot spots” within antigenic peptides showcases this more nuanced nature of TCR cross-reactivity. Within these hot spots, there is a reduction in peptide chemical and structural diversity. This permits the formation of similar, if not identical, key interatomic interactions between the TCR and peptide. Outside of hot spots, greater variability is tolerated. For example, libraries of ligands for the 2B4 and 42F3 TCRs revealed constraints on the identities of residues at a minority of positions, and structural analyses demonstrated similar interactions with the TCR binding loops were formed [88]. Similar results were found with the 1E6 TCR [39]. Such constraints were even demonstrated in the earliest structural studies of TCRs: the binding of the archetypal anti-viral TCR A6 was shown to depend on the identity of residues at positions 4 and 8 of the peptide, which were subsequently shown to play conserved interfacial roles [89].

Integrating the concepts of peptide hot spots alongside other immunological constraints permits rationalization of and even predictions about the makeup of ligands for a given TCR. Reversing the logic permits predictions about TCR repertoires based on peptide identity. However, such rationalizations and predictions are predicated on the notion that the pool of ligands for a given TCR, although large, are built around core regions of restricted structural and chemical space [90]. Yet the size and scope of such restrictions remain largely untested. It is possible, for example, that TCRs could engage
multiple pools of ligands, each defined by distinct structural and chemical cores. The presence of multiple pools could occur due to the structural adaptability present in some TCRs and their ligands or even the multi-functional chemical properties of amino acids enriched in protein binding interfaces.

Here, building upon previous work [39], we combine affinity-based selections of peptide-MHC yeast libraries, deep sequencing, and biophysical approaches to define the specificity profile of the melanoma reactive DMF5 TCR. DMF5 is a well-studied TCR, having been used in one of the first clinical trials targeting the MART1 melanoma peptide presented by the class I MHC protein HLA-A*0201 [54]. We have previously characterized the CDR loops of DMF5 as relatively rigid and static [51], which hypothetically limits the capacity with which a TCR is capable of structural adapting to multiple epitopes [83]. Despite this theoretical limitation, we obtained 1452 unique peptide sequences, many of which were seemingly unrelated and distinct from the MART1\textsubscript{26(27L)-35} sequence. Notably, our analysis determined the DMF5 TCR recognizes two sequentially distinct classes of peptides through chemically distinct mechanisms, each with their own hotspot. Our results indicate flexibility also influences cross reactivity, but in this study, the pMHC shifts and structurally adapts to the surface of DMF5. Thus, we highlight how recognition is a result of a binding event between two molecules, and both must be considered in future studies in epitope discovery, vaccine design, and immunotherapy.
4.2 Results

4.2.1 Identification of distinct classes of DMF5-reactive peptides

Yeast display, when followed by TCR tetramer staining, is a powerful technique to categorize and survey the massive repertoire of peptide antigens recognized by one TCR [39]. Recent work hypothesized that the mechanistic basis of TCR recognition depends on structural conservation of a pMHC, suggesting a TCR is limited to recognizing structurally similar peptides [39]. To evaluate this hypothesis, our collaborators provided 1452 peptide sequences derived from yeast clones stained by the DMF5 TCR.

Clustering the 1452 unique sequences by Levenshtein distance (Figure 4.1A) and filtering by read frequency identified 10 dominant peptides for further experimental analysis (Table 4.1). Although all selected peptides contained a conserved glycine (G) in position 6, surrounding residues were chemically distinct. One clade of peptides closely resembled the known MART1<sub>26(27L)-35</sub> peptide antigen, possessing a compact hydrophobic core at positions 4-7 (referred to as GXGX peptides). Surprisingly, we identified a second clade by the presence of a charged core incorporating a neighboring aspartate and arginine (DRG). Interestingly, a statistical coupling analysis [91] (Figure 4.1B) presented a possible linkage between positions 4, 5, and 10 of the peptide, further supporting the existence of distinct “classes” of peptides recognized by DMF5.
Figure 4.1 **Sequence analysis of DMF5-selected epitopes reveal distinct epitope families.** (A) Heirarchical clustering of peptide sequences reveals 10 peptide clades within two larger peptide families. (B) A covariation analysis suggest the amino acids at p4, p5, and p10 are linked.
TABLE 4.1
PEPTIDES SELECTED FOR DMF5 RECOGNITION

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Sequence</th>
<th>T&lt;sub&gt;m&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (°C)</th>
<th>K&lt;sub&gt;b&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (uM)</th>
<th>ΔG° (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MART-1</td>
<td>ELAGIGILTV</td>
<td>65.0 +/- 0.1</td>
<td>5.53 +/- 0.46</td>
<td>-7.17 +/- 0.02</td>
</tr>
<tr>
<td>Gar-1</td>
<td>SMLGIGIVPV</td>
<td>66.1 +/- 0.2</td>
<td>42.5 +/- 12.5</td>
<td>-5.96 +/- 0.09</td>
</tr>
<tr>
<td>Gar-3</td>
<td>NLSNLGILPV</td>
<td>66.3 +/- 0.1</td>
<td>7.32 +/- 0.88</td>
<td>-7.00 +/- 0.04</td>
</tr>
<tr>
<td>Gar-4</td>
<td>IMEDVGWLNV</td>
<td>55.1 +/- 0.3</td>
<td>121 +/- 12</td>
<td>-5.34 +/- 0.03</td>
</tr>
<tr>
<td>Gar-5</td>
<td>NMGGLGIMPV</td>
<td>58.6 +/- 0.2</td>
<td>11.0 +/- 1.4</td>
<td>-6.76 +/- 0.04</td>
</tr>
<tr>
<td>Gar-6</td>
<td>MMWDRGMGLL</td>
<td>45.4 +/- 0.1</td>
<td>98.9 +/- 8.7</td>
<td>-5.43 +/- 0.03</td>
</tr>
<tr>
<td>Gar-7</td>
<td>MMWDRGLGMM</td>
<td>45.2 +/- 0.1</td>
<td>31.7 +/- 2.1</td>
<td>-6.13 +/- 0.02</td>
</tr>
<tr>
<td>Gar-8</td>
<td>LMFDRGMSLL</td>
<td>50.0 +/- 0.4</td>
<td>123 +/- 16</td>
<td>-5.33 +/- 0.04</td>
</tr>
<tr>
<td>Gar-9</td>
<td>SMAGIGIVDV</td>
<td>65.8 +/- 0.1</td>
<td>202 +/- 24</td>
<td>-5.04 +/- 0.03</td>
</tr>
<tr>
<td>Gar-10</td>
<td>ILEDRFFNQV</td>
<td>46.1 +/- 0.2</td>
<td>144 +/- 20</td>
<td>-5.24 +/- 0.04</td>
</tr>
</tbody>
</table>

<sup>a</sup>Determined by differential scanning fluorimetry

<sup>b</sup>Determined by surface plasmon resonance
4.2.2 Structural divergence of DMF5-recognized peptides in context of HLA-A2

To assess the structural features of the peptides in the HLA-A2 binding groove, structure guided design techniques from the aforementioned chapter were adapted and modified to model diverse peptides in context of the MHC molecule. The models of the peptide/HLA-A2 complexes suggested the GXGX and DRG peptides adopt different conformations in the binding groove, with the DRG peptides showing more variation in the center and a greater bulge in the C-terminal half (Figure 4.2A). Analysis of the structural models suggested the large, bulky residues in peptide position three of the DRG peptides sterically prevented the sequentially conserved glycine in position six from occupying the same three-dimensional space as p6G in the GXGX peptides. This structural variability in the peptide implies that the apparent peptide specificity the DMF5 TCR has for p6G may in fact not be the result of direct TCR engagement.

To help validate the pMHC modeling and scoring, we assessed the binding of the peptides to HLA-A2 using differential scanning fluorimetry (DSF). On average, $T_m$ values of HLA-A2 loaded with a DRG-core peptide were 16 degrees lower than those of the GXGX complexes (Table 4.1). The experimental $T_m$ values correlated well with the energetic scores of the peptide/MHC complexes (Figure 4.2B), thus supporting the structural modeling and providing additional evidence of distinct peptide classes differing in sequence, conformation, and energetic stability.
Figure 4.2 **Structural modeling implies class dependent conformations.** (A) The lowest energy decoys of all DRG peptides adopted a conformation distinct from GXGX peptides. (B) Associated energy score with each decoy is correlated with experimental thermal stability measurements (R=0.87).
Unlike those in the DRG class, the molecular models of peptides within the GXGX clade all closely resembled the previously crystallized MART1_{26(27L)-35}/HLA-A2 crystal structure [54]. To confirm the structural divergence of the DRG peptides, we crystallized and solved the structure of the peptide MMWDRGLGMM bound to HLA-A2. We obtained crystals in conditions similar to other published pMHC structures [92], and we solved the structure at 2.5Å resolution in the P121 space group with two molecules in the asymmetric unit. As suggested by the structural models, the conformation of MMWDRGLGMM in the binding groove differed from that of the MART-1 decamer. In this structure, the tryptophan in position 3 is sterically preventing the peptide from adopting a backbone conformation similar to the MART1_{26(27L)-35} peptide antigen. This steric effect pushed the peptide backbone towards the α1 helix by more than 2Å. Additionally, unlike the p7 isoleucine in MART-1, the leucine at position 7 of MMWDRGLGMM was oriented towards the base of the groove, in close proximity to the usual position of Val152. This induced a displacement in the short arm of the HLA-A2 α2 helix, beginning near Val152 and continuing through His145, thus limiting the accuracy of the molecular models near the C-terminus (Figure 4.3). The displacement is maximal at Ala150, widening the binding groove by nearly 2 Å relative to the MART-1 and other peptide/HLA-A2 complexes. Thus, the MMWDRGLGMM/HLA-A2 structure confirms that the GXGX and DRG classes of DMF5 ligands differ substantially not only in chemical composition, but also in the conformations adopted and induced when bound to HLA-A2.
Figure 4.3 **Crystallographic conformation of the Gar7 peptide presented by HLA-A2 is distinct from MART1_{26(27L)-35} and molecular models.** The MHC molecule shifts to accommodate the DRG peptide (green) conformation. This was not predicted through molecular modeling energy minimization (magenta), or by other DMF5-recognized peptides (MART1 PDB: 1Jf1, blue).
4.2.3 DMF5 engages the GXGX and DRG peptide classes through distinct mechanisms

We next used biophysical and functional assays to assess if and how the differences in the GXGX and DRG peptides influence TCR recognition. To determine the implications this has on DMF5 recognition, we performed equilibrium-binding measurements via surface plasmon resonance. The binding affinities of the DMF5 TCR for each of the selected pMHC molecules were within the expected biological values for TCR:pMHC interactions, ranging from 5-300µM (Table 4.1). Furthermore, the IFNγ release of DMF5 transduced T cells interrogated with peptide-pulsed T2 cells demonstrated similar trends, exemplifying the relationship between TCR binding affinity and T cell function (Figure 4.4B). Notably, we did not observe a significant difference in binding free energy or functional preference between the distinct peptide classes (Figure 4.4A). Thus, epitopes do not need to adopt a similar conformation for a recognition event to occur by the same TCR. Instead, recognition in this instance appears to be dictated by finer distinctions in the various peptides.

Although the DMF5 TCR did not appear to display an energetic preference for either class of peptides, the binding kinetics suggested distinct modes of recognition. The kinetics were unmeasurable at 25°C, but lowering the temperature to 10°C allowed for $k_{off}$ measurements for some of the higher affinity interactions. TCR association and dissociation rates for GXGX peptides were rapid, as reported previously for the DMF5 TCR binding MART-1 [93]. In contrast, DMF5 recognized the DRG peptides with both slower off- and on-rates. For example, the TCR off-rates with peptides MMWDRGLGMM and LMFDRGMSLL were between 6- and 12-fold slower than with the MART-1 and
Figure 4.4 DMF5 Recognition of HLA-A2 Presenting Different Peptides. (A) ddG measurements of soluble proteins and T cell functional measurements show no preference for either class of peptides. (B) Binding measurements and functional measurements are closely correlated.
SMLGIGIVPV decamers. On rates were even more dramatically affected, with the rates for the DRG peptides slower by 14- to 40-fold, respectively (Figure 4.5).

Additionally, restrictions in MHC flexibility appeared to have a differential impact on DMF5 recognition. Noting the structural perturbation observed in Figure 4.3A for the MMWDRGLGMM, we introduced an A150P mutation to the HLA-A2 molecule to ‘lock’ the A2 short arm into position as reported previously [93]. The A150P only slightly inhibited DMF5 recognition of the MART1_{26(27L)-35} peptide/HLA-A2 complex, but dramatically inhibited recognition of the MMWDRGLGMM peptide (Figure 4.6). This result, together with the crystallographic data and binding kinetics, confirm that that peptide-dependant adjustments in HLA-A2 facilitate recognition of distinct peptide classes [83] and play a crucial role in TCR cross reactivity.

4.2.4 Structural interpretation of the DMF5 TCR bound to different peptides

To critically examine how DMF5 engages the two classes of peptides, we crystallized and solved the structures of the DMF5 TCR in complex with select GXGX and DRG peptides (SMLGIGIVPV and MMWDRGLGMM). We obtained crystals with conditions previously identified for crystallizing DMF5 complexes, although crystals containing the DRG-core peptide (MMWDRGLGMM) were unique in appearance, suggesting different crystal morphologies. We solved both structures in the C121 space group at resolutions between 2.4 Å and 2.15 Å, respectively. For the representative GXGX peptide
Figure 4.5 Binding kinetics differ between peptide classes. (A, C) DMF5 binding kinetics are measurably faster with GXGX peptides/HLA-A2. (B, D) DMF5 binding kinetics are relatively slower. All kinetic measurements taken at 10°C. $k_{on}$ obtained from equilibrium $K_D$ measurements at the same temperature.
Figure 4.6 **Impact of HLA\textsubscript{A150P} on DMF5 Recognition.** (A) HLA\textsubscript{A150P} only modestly impacts DMF5 recognition of the MART1\textsubscript{26(27L)-35} peptide antigen. (B) HLA\textsubscript{A150P} greatly impacts DMF5 recognition of the MMWDRGLGMM peptide antigen. (C) HLA\textsubscript{A150P} differentially impacts DMF5 recognition of MMWDRGLGMM with 1.58 kcal/mol additional energy.
A. 

Normalized Response Units vs. [pMHC] μM

- $K_D = 9.88 \pm 1.2 \mu M$
- $\Delta G^\circ = -6.82 \pm 0.04$ kcal/mol

- $K_D = 18.36 \pm 1.37 \mu M$
- $\Delta G^\circ = -6.46 \pm 0.02$ kcal/mol

B. 

Normalized Response Units vs. [pMHC] μM

- $K_D = 31.46 \pm 3.66 \mu M$
- $\Delta G^\circ = -6.14 \pm 0.03$ kcal/mol

- $K_D = 835.3 \pm 85.9 \mu M$
- $\Delta G^\circ = -4.20 \pm 0.03$ kcal/mol

C. 

$\Delta G^\circ = 0.68 \pm 0.05$

$\Delta G^\circ = -6.82 \pm 0.04$

$\Delta G^\circ = -6.14 \pm 0.03$

$\Delta G^\circ = -6.46 \pm 0.02$

$\Delta G^\circ = -4.20 \pm 0.03$

$\Delta G^\circ = 2.26 \pm 0.04$
(SMLGIGIVPV), clear electron density was present for the peptide (Figure 4.7B). The crystallized conformation is nearly identical to that predicted in the model of the free pMHC (full atom RMSD: 0.92Å) and very similar to that of the MART-1 peptide in the ternary complex with DMF5 and HLA-A2 (backbone RMSD of 0.5 Å when the HLA-A2 peptide binding domains were superimposed) [54]. Indeed, the DMF5-SMLGIGIVPV/HLA-A2 complex was virtually the same as that of the DMF5-MART-1/HLA-A2 complex, with no significant perturbations in side chain positions, CDR loops, or changes in TCR docking position (full atom RMSD: 0.9 Å). As expected, the GXGX regions of the SMLGIGIVPV and MART126(27L)-35 peptides formed many of the same interactions to the DMF5 TCR. This includes hydrogen bonds from peptide positions 2 and 3 to the Gln30 in CDR1α, and the incorporation of the same key water molecule to bridge the peptide center and the TCR [54]. Thus, the DMF5 TCR engages the GXGX peptides with a common structural solution, reflecting how the shared peptide conformation and the central GXGX hot spot facilitates cross-recognition in this case.

In contrast, the TCR-pMHC complex with the MMWDRGLGMM peptide, solved at 2.2 Å, displayed remarkably different properties. Most notably, the C-terminus of the MMWDRGLGMM peptide was “register shifted” compared to its configuration in the free pMHC, with the methionine at position 9 – as opposed to the methionine at position 10 – occupying the HLA-A2 F (or PΩ) pocket. (Figure 4.7A,C). Clear electron density was present for the first nine residues of the peptide, with feature-enhanced and Polder’s omit maps indicating that the 10th residue is exposed at the edge of the HLA-A2 binding
Figure 4.7 Structural implications of DMF5-recognized epitopes. (A) The peptide conformation of MMWDRGLGMM presented by HLA-A2:01 shares few structural characteristics with either of the DMF5-bound structures. (B) SMLGIGIVPV:HLA-A2:01 bound by DMF5 is reminiscent of MART-1 epitopes. (C) DMF5 recognition of MMWDRGLGMM induced a register shift, resulting in a previously unidentified epitope surface. 2FoFc electron density (shown as mesh) is contoured at 1σ.
groove. From a structural perspective, the TCR forces the p5 arginine side chain and the backbones of the p6-p8 residues down, pushing the Met10 side chain up and out of the groove and Met9 into the F pocket. The large change in peptide conformation is reflected in the RMSD of 3.5 Å for all peptide atoms when the bound and free HLA-A2 peptide binding domains are superimposed.

Demonstrating the malleability of the HLA-A2 peptide-binding groove, in the MMWDRGLGMM ternary complex the HLA-A2 α2 helix has returned closer to its more traditional configuration, associated with a shift of the p7 leucine back towards the center of the groove. The position of the side chain of Lys146, which has been previously described as a “lid” over the peptide C-terminus in class I MHC proteins[94], is also shifted closer to the peptide C-terminus. Given these conformational shifts in HLA-A2 occur coincidently with the register shift in the peptide, we hypothesize that the conformation induced by the MMWDRGLGMM peptide in the unbound HLA-A2 protein is more “open” and helps facilitate the dramatic peptide conformational change that occurs upon TCR binding.

To validate the observed peptide register shift, we generated two additional peptide variants. The first was a simple truncation of the MMWDRGLGMM peptide, removing the 10th residue to generate a nonameric peptide (MMWDRGLGM). The other replaced the methionine in p9 with an alanine. As alanine is a suboptimal anchor for HLA-A2, we reasoned this modified peptide would resist a register shift, with the peptide instead preferring to remain in the decameric configuration. Consistent with this reasoning, the truncated variant had almost no impact on binding affinity, while the p9A
variant almost completely ablated DMF5 recognition (Figure 4.8). Furthermore, the kinetics of the truncated peptide increased ~3-fold, further validating the structural adjustments (Supplement A.1).

4.2.5 DMF5 binds the distinct peptide classes similarly but with different energetic distributions

Compared to the MART-1 and SMLGIGIVPV GXGX complexes, DMF5 binds the register-shifted MMWDRLGMM/HLA-A2 complex such that the TCR is shifted approximately 2 Å towards the peptide N-terminus (Figure 4.9A). This rigid-body shift enables the TCR to make the same interactions between Q30 of CDR1α and the peptide N-terminal region as seen with the GXGX peptides. Despite the very different surfaces recognized, the CDR loops do not undergo any conformational changes themselves, consistent with previous determinations that the backbones of the DMF5 binding loops are comparatively rigid [83]. Overall, the RMSD for all atoms of the TCR CDR loops is 1.8 Å when the HLA-A2 peptide binding domains are superimposed, but less than 1.0 Å when the TCRs are superimposed.

Although there are no major CDR loop conformational changes, there are structural differences and sidechain rearrangements that dictate how DMF5 accommodates the two peptides. In binding MMWDRLGMM, the side chain of Asp91 of CDR3α rotates away from the peptide in order to avoid charge repulsion with the aspartic acid at p4 (Figure 4.9C). The p4Asp hydrogen bonds with Gly93 of CDR3α, mimicking a
Figure 4.8 Experimental evidence supports the register shift. Truncation of MMWDRGLGMM (red) to MMWDRGLGM had almost no impact on DMF5 binding affinity. In contrast, MMWDRGLGAM ablated DMF5 recognition.
Figure 4.9 Structural implications of DMF5 recognition. (A) No rearrangement of DMF5 CDR loops is observed when binding to different peptide classes (B) DMF5 docking orientation is skewed when binding MMWDRGLGMM compared to other GXGX peptides. (C) DMF5 utilizes a different energetic network to engage DRG vs GXGX peptides. (D) DMF5 takes advantage of many of the same interactions to engage HLA-A2 presenting different epitopes. (D) Steric limitations prevent DMF5 from recognizing the free form of MMWDRGLGMM.
role played by a water molecule present in the SMLGIGIVPV complex that links CDR3α to the backbone of pGly4. As noted in Figure 4.7C, the arginine at p5 has been pushed to the side of the interface, where it forms a water-bridged hydrogen bond with the carbonyl oxygen of Ala150 of HLA-A2, helping to explain the return of the α2 helix back to a more traditional conformation. Thus, a combination of TCR and peptide side chain movements, chemical mimicry, and HLA-A2 plasticity explain how both the highly charged DRG and hydrophobic GXGX peptide cores can be efficiently bound by the same TCR.

The crucial water molecule that bridges the peptide and TCR in the GXGX complexes is absent within the MMWDRGLGMM complex due to the position of the peptide backbone. Instead, the amide nitrogen of pGly6 adopts a similar role, forming a hydrogen bond linking the peptide backbone to Phe100 of CDR3β. Lastly, the side chain of Asn33 of CDR1β is rotated away from the interface, possibly to avoid clashes with pMet10. This need for Asn33 to shift to accommodate the register-shifted decamer may be one reason why the TCR binds the truncated MMWDRGLGM nonamer with an affinity identical to the decamer, even though the nonamer is locked in the register shifted conformation.

Although the TCR is N-terminally shifted when bound to MMWDRGLGMM as shown in Figure 4.9A, there are no substantial alterations in how the TCR engages HLA-A2. For example, signature TCR-HLA-A2 interactions involving Tyr51 in CDR2β, Arg65 in the HLA-A2 α1 helix, and a negative charge in CDR1α are unchanged between the MMWDRGLGMM and GXGX complexes (Figure 4.9D). Alterations in TCR interactions with
Arg65 have previously been shown to contribute to changes in peptide specificity [45], but this is not observed in DMF5 cross-reactivity with the DRG and GXGX peptides.

Superimposing the unbound MMWDRGLGMM/HLA-A2 complex on the bound confirms that multiple clashes help drive the register shift and conformational change in the peptide that occurs. The clashes predominantly involve the peptide backbone at pAsp4 and the side chains of pArg5, both of which clash with residues of CDR3β. The most severe clashes involve pArg5, which occupies the same volume as the backbone of Gly101β and Thr102β (Figure 4.9E).

To study how the DMF5 TCR can energetically accommodate the structural differences in the two classes of peptides, we returned to the structural modeling and scoring used in evaluating the pMHC models and previously in TCR design. Accounting for the peptide conformational change, ~15% more surface area is buried in the MMWDRGLGMM complex than the SMLGIGIVPV complex (2274 Å² compared to 2569 Å²), all attributable to polar surface (TABLE 4.2). As anticipated from the charged character of the MMWDRGLGMM peptide, non-specific burial of hydrophobic surface area can therefore not account for the ability of the TCR to engage the two classes of peptides, as has been suggested in other studies of TCR cross-recognition [19]. Computing differential interaction energies within the interfaces showed instead that in the MMWDRGLGMM complex, stronger and more focused interactions are present near the center of the peptide and Gln30 of CDR1α and Phe100 of CDR3β, compared to weaker, more distributed energies in the SMLGIGIVPV and MART-1 complexes (Figure 4.10C). The stronger energetics within the DRG complex can explain the slower TCR dissociation
TABLE 4.2
INTERFACE CHARACTERISTICS OF DMF5 COMPLEXES

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Sequence</th>
<th>Interface Component</th>
<th>Hydrophobic buried SASA (\text{(Å}^2))</th>
<th>Lennard Jones (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MART-1</td>
<td>ELAGIGILTV</td>
<td>Peptide</td>
<td>182.03</td>
<td>-8.19</td>
</tr>
<tr>
<td></td>
<td>HLA</td>
<td></td>
<td>492.65</td>
<td>-17.70</td>
</tr>
<tr>
<td>Gar-1</td>
<td>SMLGIGIVPV</td>
<td>Peptide</td>
<td>187.61</td>
<td>-6.71</td>
</tr>
<tr>
<td></td>
<td>HLA</td>
<td></td>
<td>472.83</td>
<td>-18.29</td>
</tr>
<tr>
<td>Gar-7</td>
<td>MMWDRGLGMM</td>
<td>Peptide</td>
<td>135.82</td>
<td>-10.03</td>
</tr>
<tr>
<td></td>
<td>HLA</td>
<td></td>
<td>440.02</td>
<td>-13.65</td>
</tr>
</tbody>
</table>
Figure 4.10 Lennard Jones potentials highlight the network of interactions between DMF5 and pMHC. (A-C) Surface representations of the pMHC (left) and DMF5 TCR (right) from the view of their respective binding partner. Peptides are labeled, and the Lennard Jones potentials are highlighted in red, with the intensity linearly correlated to the strength of interaction.
kinetics, whereas the conformational gyrations in the peptide and HLA-A2 are likely related to the slower association kinetics.

4.2.6 Applying computational design to improve epitope discovery

Although yeast display is a powerful tool for rapid protein engineering, limitations in library design and random combinations reduce the overall effectiveness when searching for the most energetically favorable event. In theory, in silico techniques can supplement the outcome of a directed evolution experiment. Indeed, advances in computing power are beginning to allow for the massive throughput required for adequate epitope screening. To identify the most energetically favored peptide combinations recognized by the DMF5 TCR, we performed a small proof of concept combinatorial screen limited to 6,048 GXGX peptides. For simplicity, we restricted TCR facing residues to amino acids observed in the yeast display screen and left MHC buried residues static (Figure 2.3). These sequential restrictions also influenced conformational sampling: no modeled peptides diverged from the previously identified class-specific crystallized conformation. We selected four of the most favored peptides for binding affinity measurements with the DMF5 TCR. In solution, each of the computationally identified peptides presented by the HLA-A2 demonstrated strong to moderate affinity for the DMF5 TCR (Figure 4.11), and we successfully identified a peptide that improved interactions to the DMF5 TCR, resulting in a high affinity interaction. Thus, since we identified additional epitopes with our admittedly limited screen, many more epitopes
Figure 4.11 Computationally selected peptides are all recognized by DMF5. Four peptides associated with the lowest energy TCR:pMHC models were selected and tested in solution for DMF5 recognition.
favored by DMF5 likely exist. We attempted a similar screen with the DRG peptides, but all predicted peptides did not induce a DMF5 binding response. This could be a result of numerous factors, but the most likely is that a structural modeling approach can at best estimate enthalpic contributions. The biophysical implications of entropy and flexibility are notoriously difficult to model and predict, and likely influenced our predictions for this class of peptides.

4.2.7 Combining sequence and structural information for biological relevance

In the current technological stages, either through directed evolution or through rational design, it is impossible to sample all possible peptide epitopes. Thus, the information obtained in the aforementioned experiments must be extrapolated to obtain potential cross reactive, naturally occurring epitopes in the human proteome. Previously, Birnbaum et al. described an approach to screen human protein databases for epitopes with sequence similarity to the epitopes obtained through yeast display libraries [39]. This approach was moderately successful, correctly identifying 6/7 human peptides which induced T cell proliferation. We applied this substitution matrix to our DMF5 libraries, and identified 43 possible human peptide targets. We next modeled each of these peptides in context of HLA-A2 and DMF5, and observed a moderate correlation between frequency score and structural score (R = 0.68, Figure 4.12A), emphasizing the energetic relationship between the two approaches. Because training data for the substitution matrix did not include any covariation linkage, the structural modeling flagged false positive GXGX/DRG
Figure 4.12 Correlation between JMBLAST and the energy score of structural models leads to identification of true and false positives. (A) A correlation between the sequence dependent JMBLAST score and structural energy score is observed with few outliers. Outliers predicted to be false positives are highlighted in red X’s, and predicted true positives are highlighted with green stars. (B) All six selected peptides were correctly labeled as either true positives (DMF5 binding response) or false positives (no DMF5 binding response).
‘hybrid’ epitopes with an unusually high score. By combining these bioinformatics and structural approaches, we were able to quickly eliminate false positives and identify a human-derived peptide that induced both a binding and functional response (Figure 4.12B).

4.3 Discussion

Immunology has long grappled with the limited-specificity paradigm of TCRs, as TCRs require the capacity to recognize a multitude of antigenic peptides while at the same time be specific enough to discriminate self vs non-self antigens. The general mechanism of αβ TCR cross reactivity appears to stem from peptide molecular mimicry, where a single TCR may recognize seemingly unrelated peptides that share enough chemical and structural similarity [88], although recognition of nonhomologous antigens certainly occurs to varying degrees in the TCR repertoire. However, such examples are surprisingly rare and some receptors are bound to have a greater degree of promiscuity. In this study, we show how even a relatively well studied TCR possesses the ability to cross-react with unrelated antigens, suggesting degenerate epitope recognition may be more common than previously assumed. With this insight into TCR recognition, we demonstrate a requirement for exceptional care when designing TCRs for clinical use.

By leveraging large amounts of experimental data via direct binding of pMHC to TCR, we find that unique structural solutions, in addition to molecular mimicry, allow the clinically relevant DMF5 TCR to sample and recognize a diverse set of epitopes. In
hindsight, this is not entirely surprising: the ability of unrelated TCRs to engage the same antigen highlights some of the structural solutions available to TCRs. Thus, it is not unreasonable to assume two epitopes contain distinct combinations of amino acids that engage the same TCR in distinct ways.

An important implication of our findings is that flexibility of the pMHC, and not just the TCR, can drive polyspecificity. As seen in the A6 TCR, flexibility of the CDR3b loop influences the degree of observed cross reactivity. In contrast, DMF5 is a relatively rigid TCR with rigid CDR loops, which has implications for specificity. However, as noted by Borbulevych [93], the pMHC is also a very dynamic molecule. The structures of Gar7 highlight the extent with which this may occur. Although structurally, the DMF5 TCR itself remained unchanged, sequentially unique peptides melded to the surface of the TCR even if initially incompatible. Small motions in the MHC molecule facilitated this melding, and we observed how restricting flexibility in either the MHC molecule or peptide inhibited DMF5 recognition.

This study highlights the difficulty in predicting the specificity profile of any particular TCR. Identifying the hotspot of one epitope can only identify epitopes that share the same structural characteristics. In theory, structural modeling can sample additional space and identify the compatible epitopes, although, as we show here, flexibility substantially increases the degrees of freedom. Indeed, we failed to predict how the motions of the MHC molecule would influence peptide presentation or DMF5 recognition of the DRG class of peptides. However, once we elucidated the mechanisms of recognition of the two peptide classes, predicting recognized peptides through rational
design and structural modeling became substantially more feasible. As the technology grows, develops, and computational resources become more readily available, structural modeling may surpass experimental methods when defining the peptide specificity of a TCR.

The naturally occurring peptides in this study highlight the strengths of combining a structural based approach with deep sequencing. The JMBLAST approach previously described [39] is extremely effective at identifying epitopes that share sequence similarities with the peptides pulled from the yeast libraries. Modeling these epitopes and scoring the energetic interactions emphasize that, in general, epitopes are selected from the yeast libraries based on an energetically favorable binding event with a TCR. However, it is not feasible to screen all epitopes in vitro, and oftentimes it is impossible to determine what is not possible from an experimental standpoint. Thus, structural modeling can ‘fill the gaps’ from the directed evolution experiments, and has the potential to advance our understanding of T cell recognition and the complex mechanisms associated with immune-mediated diseases.
CHAPTER 5:

STRUCTURAL FEATURES OF IMMUNOGENIC EPITOPES

5.1 Introduction

Peptide vaccines represent another branch of immunotherapy and have been presented as an alternative or complement to adoptive cellular immunotherapy [95]. Whole tumor sequencing often results in the identification of thousands of tumor-specific “neoepitopes,” but discriminating immunologically recognized neoepitopes and optimal targets for vaccine development is a current challenge. Here, a novel technique to identify potentially immunogenic neoepitopes is presented, and uniquely focuses on structural comparisons to predict TCR recognition.

Previous studies have been able to correlate improved neoepitope stability in the MHC binding groove with increased immunogenicity and tumor rejection in mice [42]. Although this technique was successful when comparing N- or C- termini ‘anchor-modified’ neoepitopes, success was limited when mutations occurred in the central regions of the peptide. This has led to the hypothesis that an epitope must have characteristics beyond the ability to bind with an MHC molecule. Others have noted an enrichment of solvent exposed hydrophobic and/or aromatic residues in viral
immunodominant epitopes [63]. Structurally, many epitopes, mainly nonamers, adopt similar conformations when presented by an MHC molecule, facilitating the ease of predicting which amino acids in an epitope would be “seen” by a TCR [96]. This has led to the development of epitope prediction servers, which combine the anchor preferences of HLA alleles with the chemical properties of predicted TCR-facing residues [63].

These initial prediction servers emphasize amino acid preferences oftentimes without considering the impact of neighboring residues or structural perturbations [41]. Indeed, the most widely used server, netMHC, is a neural network trained to predict the binding affinity of an epitope to a given MHC molecule by amino acid sequence alone. Although generally successful, the training data set used by netMHC is of varying quality and included a large amount of data influenced by biological factors [41]. For example, while many epitopes may be biophysically compatible with an MHC molecule, amino acid composition of the training set will almost certainly bias results. Additionally, netMHC fails to account for minor structural perturbations. As such, longer epitopes with greater degrees of conformational freedom often give erroneous results.

Thus, structural information may improve the current sequence based approaches for epitope prediction. Structural interpretations provide greater depth regarding why certain residues are preferred, and can predict the fine structural changes between epitopes and the associated biophysical implications. For example, structural information clearly implicates the structural surface and hydrophobicity of the p2 and F pockets of HLA-A2 driving the preference for select ‘anchor residues’ in the corresponding epitope. Therefore, a structural model can provide a template for which to assign energetic values
without the biologically imparted biases. However, despite the clear advantages of incorporating structural information, accurately modeling peptide epitopes in context of the MHC binding groove remains a challenging task [96].

Here, we observe structural features from two distinct data sets correlate with tumor rejection in mice [42] and clinical survival rates in human patients after treatment with the checkpoint-blockade inhibitor, ipilimumab. Despite the promising observations, the results suffered from the extremely small mouse data set and the ambiguous nature of clinical data. Thus, we collected a third data set in collaboration with the University of Oklahoma to develop an accurate immunogenicity prediction model emphasizing structural features.

5.2 Results

5.2.1 Correlation of structural features to immunogenicity and tumor rejection in mice

Data from Duan et al. identified 11 neoepitopes sequenced from mouse tumors. Further unpublished work showed that 5 of the 11 neoepitopes resulted in tumor rejection when used to immunize BALB/c mice (Supplement B.1)[42]. To gain structural insight into these epitopes, we modeled each epitope pair using similar techniques as described in the previous chapters, but with greater conformational sampling. Using a binary criterion of tumor rejection/no tumor rejection, we constructed receiver operating characteristic (ROC) plots for various classifiers. As expected, the previously described Differential Agretopic Index (DAI) [42] failed to correlate with immunogenicity, as the
predicted stability of nearly all neoepitopes was similar to the wild type. Surprisingly, we
found the difference in Talaris2013 scores of the structural models to be the strongest
predictor of tumor rejection, with higher scoring neoepitopes relative to wild type more
likely to initiate tumor rejection (Figure 5.1A). An explanation for this result can be seen
by calculating the interaction energy: $\Delta G_{\text{int}} = G_{\text{complex}} - (G_{\text{pMHC}} + G_{\text{TCR}})$ [76]. The described
equation suggests higher energy TCR and pMHC molecules will have a tendency to form
stronger interaction energies if they come together favorably in a complex. However,
generalizing our observations at this stage was difficult due to the limited data set.

5.2.2 Neoepitope structural features are good predictors of patient response to
ipilimumab

Although initial results are promising, the above work investigated only a small
data set of 11 neoepitope/wild-type pairs. Recent work carried out whole exome
sequencing on 110 melanoma patients treated with ipilimumab, a monoclonal antibody
that disrupts the inhibitory CTLA-4 checkpoint of the immune system [58]. This study
identified over 14,000 neoepitope/wild-type pairs, and a high ratio of netMHC-favored
peptides was found to be better correlated to patient survival than overall mutational
load (unpublished). To expand on this observation, we modeled each neoepitope/wild
type pair (METHODS) and compared structural features to clinical response. To minimize
the mutational load disparity between patients, we averaged the energetic scores of each
Figure 5.1 Structural modeling detects characteristics of immunogenic peptides. (A) The differential energy scores of structural models of WT/neoepitope pairs better correlates with immunogenicity than the sequence based DAI. (B) Representative models of a WT peptide and an immunogenic neoepitope, highlighting the impact of a solvent exposed tryptophan.
patient’s neoepitopes into a singular aggregated score. Although this approach likely
obfuscated elements of unique patient specific elements, we were able to identify several
trends. Consistent with previous observations others and we have made [42], if a
neoepitope was the result of an anchor modification, the new mutation must improve
the repulsive energy within the structural model. Conceptually, these neoepitopes may
be better presented by HLA-A2 and thus be a potential source of immunogenicity.
Additionally, patients with a high solvation score, implying a greater degree of solvent
exposed hydrophobic surface area, were also more likely to be associated with survival
and/or clinical benefit. We used these observations to train a simple regression model
that could predict patient outcomes based on neoepitope composition with 83% accuracy
(Figure 5.2), although the model is likely over-fit without a corresponding testing data set.
Thus, structural information can help elucidate features of immunogenicity beyond pMHC
stability.

5.2.3 Building a neural network to predict epitope immunogenicity

The observations noted above were useful, but it is difficult to develop a
quantifiable model from clinical data where factors beyond neoepitope immunogenicity
may influence patient outcomes. Therefore, we sought a data set based on more
quantitative data. The IEDB has substantial records for immunogenic peptides, including
ELISpot and IC50 data, but little data on ‘self’ peptides to which T cells are tolerized to
during thymic selection. Thus, we turned to a proteomics based approach to eliminate
Figure 5.2 **Survival can be predicted from neoepitope features.** Receiver operating characteristic plots of predicted survival rates trained to various neoepitope classifications.
the selection bias within the IEDB. Our collaborators developed a novel technique to sample biologically processed peptides presented by HLA-A2 via mass spectrometry and proteomics, and provided us the sequences of these ‘self’ peptides. To minimize modeling errors, we focused exclusively on nonameric peptides, resulting in a data set of 2712 ‘self’ peptides and 155 immunodominant peptides. The final data set was completed with 1044 HLA-A2 incompatible peptides selected from netMHC training sets [59], with a measured affinity of >50,000nM. Aside from the typical anchor residue preferences, hydrophobic residues were more enriched in the immunogenic peptides than the HeLa eluted ‘self’ peptides (Figure 5.3), particularly in the typically solvent exposed peptide positions 4,5,7, and 8 (52% vs 42%, p<0.01) (Supplement B.2).

Neural networks have the capacity to integrate higher order correlations into prediction scores, unlike the linear prediction methods used above. For example, a given peptide must first be capable of binding an MHC molecule before immunogenicity categorization. To capture this and other higher order correlations, we constructed a neural network to predict the immunogenicity of a peptide given a structural model. Ideally, this approach would provide finer resolution than similar, sequence based immunogenicity prediction servers, and could facilitate peptide vaccine design.

After generating structural models of each peptide in this data set in context of HLA-A2, we extracted 117 energetic terms reporting on the features of the corresponding peptide model. After cross validation, we retained 81 terms for neural network inputs, which resulted in an average cross-validated AUC of 0.69. After training, the final neural
Figure 5.3 *Residue distributions of peptides in SKYNET training pools.* (A) Immunogenic peptides. (B) Eluted HeLa peptides. (C) Peptides with measured IC50 >50,000 nM affinity for HLA-A2.
network, termed SKYNET3.0, adequately classified all peptides used in training as immunogenic or not immunogenic with a total AUC of 0.73 (Figure 5.4).

5.2.4 Performance of SKYNET3.0 on NY-ESO variants and comparisons to alternative techniques

To test the performance of SKYNET3.0 on data not used in the training and validation procedure, we deployed SKYNET3.0 on a published data set of NY-ESO peptide variants. The stability and immunogenicity of these peptides was previously characterized [97], and provides SKYNET3.0 with a panel of similar in sequence peptide epitopes with dramatically distinct immunogenicity outcomes. SKYNET3.0 did not outperform the reported, experimentally determined ‘refold score’ (AUC 0.89), although it did perform similarly with the IEDB immunogenicity tool with an AUC of 0.69 and 0.73, respectively (Figure 5.5B, C). The two methods were also positively correlated, with a correlation coefficient of 0.67 (Figure 5.5F). Thus, SKYNET3.0 reports similar information as the IEDB immunogenicity tool, despite different approaches to encode peptide information and distinct training sets.

5.3 Discussion

The identification of neoepitopes and subsequent proposals for peptide cancer vaccines pose an exciting proposition to the field of immunology and cancer research.
Figure 5.4 Sensitivity of SKYNET3.0 when predicting training set immunogenicity Receiver operating characteristic plots of between predicted and observed immunogenicity of the trained SKYNET3.0 algorithm redeployed against the entire original training set after training and cross validation.
Figure 5.5 **Performance and comparison of SKYNET3.0 and other predictors of immunogenicity on a data set of NY-ESO variants.** (A-C) ROC plots of different techniques predicting the immunogenicity of NY-ESO variants. (A) The experimentally determined refold score measuring stability of each NY-ESO variant. (B) The IEDB immunogenicity score based on a sequence-trained neural network. (C) SKYNET3.0. (D-F) Comparisons of different methods to each other, highlighting the reporting of similar epitope features.
However, this requires a deep understanding of the relationship between peptide presentation and T cell response and necessitates new approaches for unbiased antigen discovery. By leveraging distinctive atomic features of immunogenic epitopes, we trained a neural network that predicts the ability of a peptide to elicit an immune response after first considering the ability of the peptide to first bind HLA-A2 and then considering if the presented structural features resemble those of generic immunogenic epitopes.

Our initial observations highlight the importance of epitope structural features when considering immunogenicity. In addition to compatibility with the MHC molecule, a given immunogenic peptide will also likely demonstrate features compatible with traditional views of protein-protein interactions. Due to the complexity of the pMHC interface, these features may not be readily apparent through sequence alone. Thus, with proteomics and structural modeling, we generated a rich data set for identifying key features of immunogenicity beyond simple sequence-level associations. Accordingly, SKYNET3.0 incorporates several important attributes of immunogenic epitopes. Optimal epitopes tend to have (1) compatible ‘anchor residues’ in positions 2 and 9 of the peptide, (2) a structure compatible with the chosen MHC (in this case HLA-A2), and (3) an exposed surface enriched in hydrophobic residues.

SKYNET3.0’s use of structural features adds several key advantages to the field of epitope discovery. First, although proteolytic processing may bias the sequence information of available MHC-associated peptides, SKYNET3.0 searches for compatible structural features rather than sequence similarity. For example, cysteine appears relatively rarely in the p9 anchor position in the IEDB database of 9-mer HLA-A2
associated epitopes (0.5%). As such, the known tumor antigen NY-ESO (SLLMWITQC) is predicted to be a poor HLA-A2 binder by netMHC (>500nM) and other sequence-based prediction algorithms. However, what is presumed to be a biological restraint does not hinder SKYNET3.0, which adequately predicts NY-ESO to be an immunogenic epitope. Consistent with this line of reasoning, SKYNET3.0 is not restricted to the standard 20 amino acids. These two unique features greatly expand the possibilities of custom designed peptide vaccines. However, perhaps the most valuable aspect of this approach is the generation of structural models for each characterized epitope. This provides tangible feedback for any given peptide sequence, allowing structural biologists and immunologists alike to identify key elements of immunogenicity, in conjunction with the SKYNET3.0 score.

Despite the apparent advantages, SKYNET3.0 did not outperform the current IEDB prediction tool for immunogenicity. There are likely several explanations for this, such as the limited modeling protocol, the focus on nonameric peptides, and perhaps even an incomplete feature map describing different physical parameters (i.e., poor solvation model). However, protein structural modeling is in its infancy, and will continue to improve as a science over time. In contrast, primary structures encodes limited information, and algorithms dependent on this information have likely reached their peak in terms of predictive power. Thus, considering the shortcomings of both techniques in their current forms, we predict structure-based prediction models to be far superior in the near future.
CHAPTER 6:
CONCLUSIONS

6.1 Overview

T cell engagement with antigen presenting cells is a particularly unique feature of the adaptive immune system. The weak affinities and polyspecificities of T cells is at first counterintuitive to the perceived requirement for precision within the immune system. However, the obvious success of the immune system when defending against foreign pathogens is a testament to the elegance with which the immune system has evolved to take advantage of these principles.

The focus of these investigations have revolved around understanding how TCRs take advantage of well-understood biophysical principles to recognize the composite pMHC surface, with applications to TCR design, vaccine design, and epitope discovery. Although the binding energetics of multiple TCRs have been analyzed extensively, the lack of a singular mechanism underpinning aspects of TCR recognition such as MHC restriction and polyspecificity has hindered the development of a generalized binding model. Indeed, current evidence would suggest a generalized mode of TCR binding might not even exist. However, the atomic level energetic analyses provided by structural modeling can give
additional information regarding TCR-pMHC binding mechanisms. Here, we trained the
ergetic and structural data of computational models to a large sampling of
experimental data in an effort to advance our own understanding of the biophysical
drivers of TCR-pMHC recognition.

6.2 Manipulating the relationship between structure and function for therapeutic benefit

Membrane composition, co-receptors, and supramolecular architectures all provide examples of how T cell functionality can be influenced independently of classical biochemical parameters that influence the number of ligated receptors (i.e. affinity and receptor/ligand concentrations). Because these complexities superimpose on receptor binding in determining T cell function, functional outcomes scale imperfectly with TCR binding affinity measured in solution. Indeed, many outliers have been noted over the years and both high and low thresholds are believed to exist [18, 19, 98]. Nonetheless, numerous experiments have demonstrated how changes within a TCR-pMHC interface lead to changes in binding affinity and corresponding changes in functional readouts [18, 19, 98]. Thus, the TCR is a prime target in efforts to manipulate T cell functional responses for cell based immunotherapies.

However, it is clear that simply improving affinity is not sufficient to improve T cell function. Maintaining specificity of the TCR is also required for genetically engineered T cells, in order to limit the extent of cross-reactive side effects. The composite nature of the pMHC ligand presents an obstacle to traditional protein engineering techniques, but
is ideally suited for the precision associated with structure guided design methodology. By adopting such a structure-guided strategy, we highlight how these techniques are very successful at explicitly considering the contributions to $\Delta G^\circ$ of typical structural interpretations of atomic interactions (van der Waals interactions, hydrogen bonds, burial of hydrophobic surface area, etc.). Thus, armed with the ability to predict with high accuracy the impact of any given mutation, we can begin designing TCRs with targeted affinity improvements.

6.3 The structural basis for TCR cross reactivity

When engineering TCRs, we must also acknowledge TCRs are inherently not highly specific. As has been recognized for some time, TCR cross-reactivity is fundamental to the immune system. Yet cross-reactivity is not random, but driven by the fact that for any one TCR, many peptides will be compatible. Achieving such a structural/energetic alignment will not always be fully obvious or predictable from sequence comparisons, as structural, chemical, and motional similarities influence TCR engagement. Moreover, small regions of the ligand, i.e., hot spots, may contribute the most dominant interactions. These hot spots, together with structural and physical considerations, can explain long-standing observations that TCRs can be sensitive to small perturbations in one region of the peptide, while tolerating more dramatic changes elsewhere.

However, our data demonstrates that TCR specificity is not solely restricted to epitopes with shared hot spots. The complexity of TCR:pMHC interfaces allow for
multiple structural solutions to exist, with some epitopes differing in key structural and chemical characteristics. Regardless, this apparent increase in cross reactivity is compatible with early estimations of the scope of TCR specificity profiles. Consider that conservation of a TCR-specific hotspot can yield over a million compatible peptide permutations, with very similar structural features. Now, consider the existence of another peptide class with its own unique hot spot, yielding yet another million compatible TCR epitopes. However, because these epitopes share different, sometimes overlapping hot spots, features from one epitope class are often not compatible with the other. When combined into a single epitope, such a ‘dual hotspot’ epitope may be unable to elicit a TCR response. In the example we provide, DMF5 recognition of a peptide with a DRG central motif requires the c-terminus to extend beyond the HLA-A2 binding groove. Confining the DRG peptide within the binding groove and forcing the peptide to adopt a conformation similar to the GIGI class of peptide abolishes TCR recognition. Thus, the existence of multiple classes of TCR compatible epitopes only extends the TCR specificity profile linearly instead of combinatorically, limiting the extent of TCR recognition to just a few million peptides.

Most efforts to identify TCR-compatible epitopes, usually to avoid cross-reactive side effects with therapeutic TCRs, stem from hot spot comparisons within the human proteome [39]. A single known epitope is often the basis of comparison, and it is thus impossible to search for alternative hot spots. Therefore, there exists a need to rapidly screen a TCR for structural epitope solutions. Although the method used by Garcia et al. is powerful, the limitations of yeast display hinder this method. However, the massive
degrees of observed freedom in both TCR and pMHC greatly hinder the ability of structure-guided design to act as a solution with the current technological capacity. As we highlight here, the best approach may be a hybrid method, where directed evolution is useful for broad searches of hot spot compatibility, and further expanded \textit{in silico} via structural analysis and design.

6.4 Structural features of immunogenic epitopes

As alluded to above, a T cell signaling response in response to an antigen is dependent on many codependent variables. T cells are mediated by a variety of signaling molecules and are sensitive to environmental effects. Additionally, co-receptors such as CD8, in addition to the TCR and pMHC, are often required to form the T cell signaling complex. Regardless, the first step towards initiation of an adaptive immune response requires a response to a binding event between TCR and pMHC. Although there have been significant efforts to define the nature of a TCR:pMHC interaction, reports have failed to identify an overarching signal driving pMHC recognition. This implies TCR:pMHC interactions are subject to many of the generalizations made over the years about protein:protein interactions. Specifically, binding interfaces tend to be enriched in hydrophobic residues with minimal electrostatic interactions. Indeed, others and we have noted that viral and immunogenic epitopes tend to be significantly more hydrophobic than those associated with ‘self’ epitopes are.
The reason for this is unclear: Is this enrichment a result of an alternative epitope processing pathway, or more a characteristic of the evolved divergences between vertebrates and viral genomes? Although our approach does not address these questions, these characteristics are a prime example of the features captured by structural modeling. Thus, a structural approach may provide more insight than a pure bioinformatics strategy.
APPENDIX A:

ADDITIONAL INFORMATION FOR DMF5 CROSS REACTIVITY
Figure A.1 Binding kinetics between DMF5 and ELAGIGILTV, MMWDRGLGMM, and MMWDRGLGM
### TABLE A.1

STRUCTURE REFINEMENT STATISTICS

<table>
<thead>
<tr>
<th>Construct</th>
<th>MMWDRGLGMM Free</th>
<th>MMWDRGLGMM Bound</th>
<th>SMLGIGIVPV Bound</th>
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APPENDIX B:

ADDITIONAL INFORMATION FOR EPITOPE CHARACTERISTICS
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<sup>a</sup>Neopeptides derived from MethA mouse tumors

<sup>b</sup>Determined by difference in netMHC3.0 scores
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60. McMurtrey, C., et al., Toxoplasma gondii peptide ligands open the gate of the HLA class I binding groove. Elife, 2016. 5.


