ENGINEERING AND CHARACTERIZATION OF HUMAN SINGLE-CHAIN T CELL RECEPTORS

BY

SHEENA N. SMITH

DISSE TATION

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Doctoral Committee:

Professor David M. Kranz, Chair
Professor Wilfred A. van der Donk
Professor Robert B. Gennis
Professor Edward J. Roy
ABSTRACT

All nucleated cells display a sampling of their protein contents in the form of short 9-10 amino acid peptides bound to a product of the major histocompatibility complex (MHC) on the cell surface. This class of MHC-restricted antigens provides the broadest class of potential disease targets, as the peptides displayed are derived from the full antigenic repertoire of the cell, including intracellular, secreted, and cell-surface proteins. These peptides can be from endogenous “self” proteins, or they can result from viral infection or transformation to a cancerous state. Activation of the cell-mediated immune response begins with the binding of “foreign” MHC-restricted antigens by T-cell receptors (TCRs) with an affinity above the threshold for activation by the T cells. Like antibodies, TCRs are generated with large diversity in order to have some TCRs that accomplish this antigen recognition, but unlike antibodies TCRs do not undergo somatic hypermutation and therefore have relatively low affinities ($K_D = 1$-100 $\mu$M). The generation of higher-affinity TCR variants provides a useful approach to improve targeting of cancerous cells, and to study the principles of TCR recognition and T cell triggering.

TCRs are $\alpha\beta$ heterodimers that interact with peptide-MHC (pepMHC) through six complementarity determining region (CDR) loops. The relative positions of these loops is conserved, such that the regions derived from the germline encoded regions of the TCR (i.e. CDR1 and CDR2) are positioned primarily over the MHC helices whereas the diverse regions derived from the junctions of somatically rearranged gene segments (i.e. CDR3) are positioned primarily over the peptide. Over the last 15 years, the Kranz
lab has developed a strategy to engineer stable, high affinity TCRs using yeast display of TCRs in a single-chain format (scTCR) that consists of the variable regions of the α and β chains connected by a flexible linker (Vα-linker-Vβ or Vβ-linker-Vα). The focus of this dissertation is the engineering of human high affinity scTCRs for the targeting of cancer antigens, and the use of high affinity scTCRs for understanding the principles MHC-restriction and T cell specificity.

In Chapter 2, a human scTCR specific for the melanoma cancer antigen Melan-A/MART-1 was engineered for improved stability and affinity by yeast display. The high affinity scTCR was expressed as a soluble protein in *E. coli* for MART-1/HLA-A2 binding studies and detection of the specific antigen on the surface of human antigen presenting cells (APCs). This TCR, called T1-S18.45, expresses the stable Vα2 region that is also expressed by two viral-specific TCRs, A6-X15 and 868-Z11, previously engineered by our lab. Using this panel of three high affinity scTCRs, engineered for high-affinity in CDR3 loops, mutational analysis was performed at residues in the CDR1α and CDR2α, testing a prominent hypothesis that holds that there are several evolutionary conserved residues in TCR variable regions that contact MHC.

In Chapter 3, a human scTCR specific for Wilms’ Tumor Antigen-1 (WT-1) was engineered for improved stability and affinity by yeast display through a multi-step affinity maturation process. This scTCR was also expressed in *E. coli* as a soluble scTCR and used to detect WT-1/HLA-A2 on the surface of human antigen presenting cells. The WT-1 antigen is considered a promising therapeutic target for leukemia as
well as various solid tumors, and as a result the engineered receptor has been further pursued in adoptive cell therapy models.

The conventional approach to engineering high affinity scTCRs to date has required the isolation of specific T cell clones and their clonotypic TCRs prior to in vitro engineering. In Chapter 4 a strategy using in vitro, directed evolution of a single TCR to change its peptide specificity is described. The approach, avoids the need to isolate T cell clones for each MHC-restricted antigen of interest. The human TCR A6, that recognizes the viral peptide Tax in complex with HLA-A2, was converted to TCR variants that recognized the cancer peptide MART-1/HLA-A2 through mutagenesis and selection. Mutational studies and molecular dynamics simulations identified CDR residues that were important in this specificity switch. In addition, TCR variants that exhibited broad cross-reactivity with different peptides were identified, providing opportunities to examine in an unprecedented way the basis of peptide specificity. These finding thus provided a new platform in which in vitro engineering strategies alone could be used to isolate designer TCRs with desired specificities.
To my parents, Cory & Rosa Leland, for inspiring me to pursue my passions and cultivating my scientific curiosity and love for learning.
ACKNOWLEDGEMENTS

The completion of my graduate studies and the work that is subject of this dissertation could not be possible without the support of my advisors, colleagues, collaborators, mentors, family, and friends. First and foremost I would like to thank my advisor, Dave Kranz, for allowing me to work in his laboratory and providing a tremendous amount of guidance relating to my research. It is an extremely exciting time to be working in the field of cancer immunotherapy, and I am very fortunate Dave allowed me to join his laboratory and work on such fascinating and significant projects. His extensive knowledge, experience, and patience over the course of my PhD work have been instrumental in my development as a scientist, and I am very honored to have studied in his laboratory.

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during the course of my PhD work. I would also like to acknowledge the newest member of our group, Dan Harris, whom I have had the pleasure to train and work with during the last couple years in the lab. Dan shows great promise, and I am very excited that much of the continuation of my work is in his competent hands. Finally, I am grateful for the past members of the Kranz lab for their contributions to the body of knowledge of our group that has laid foundation for the current work in the lab, and for developing many of the techniques and approaches that have made my work possible.

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Throughout my graduate career I have gotten the opportunity to collaborate with many highly skilled scientists. Internally at the University of Illinois I had the privilege to work with Emad Tajkhorshid and two of his students, Yuhang (Steven) Wang and Javier Baylon, who provided molecular dynamics simulations that are described in Chapter 4. Additionally, I have worked with several external collaborators that I would like to thank, including Wolfgang Uckert at the Max-Delbrück-Center for Molecular Medicine (Berlin, Germany) and his former student Daniel Sommermeyer (now at the Fred Hutchinson Cancer Research Center, Seattle, WA) as well as Helga Bernhard at Medizinische Klinik V (Darmstadt, Germany) for isolation of the MART1/HLA-A2-specific T cell clones and TCR genes described in Chapter 2. Also, Philip Greenberg and Thomas Schmitt at the University of Washington for the isolation of the WT1/HLA-A2-specific T cell clones and TCR genes described in Chapter 3. Finally, I would like to thank Brian Baker, Sydney Blevins, and Nishant Singh at the University of Notre Dame as well as Kurt Piepenbrink (now at The University of Maryland Medical School) for contributing surface plasmon resonance (SPR) measurements of high affinity TCRs as well as performing initial work in solving crystal structures of high affinity TCRs (Chapters 2-4).

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2C</td>
<td>Mouse T cell that recognizes SIY/K&lt;sup&gt;b&lt;/sup&gt;, QL9/L&lt;sup&gt;d&lt;/sup&gt;, and dEV8/K&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-1BB</td>
<td>Co-stimulatory molecule; also known as CD137</td>
</tr>
<tr>
<td>454</td>
<td>High-throughput DNA sequencing system by Roche Ltd.</td>
</tr>
<tr>
<td>868</td>
<td>Human TCR that recognizes SL9/HLA-A2</td>
</tr>
<tr>
<td>868-Z11</td>
<td>Engineered human scTv that recognizes SL9/HLA-A2 with high affinity</td>
</tr>
<tr>
<td>A6</td>
<td>Human TCR that recognizes Tax/HLA-A2 and Tel1p/HLA-A2.</td>
</tr>
<tr>
<td>A6-c134</td>
<td>Engineered human TCR that recognizes Tax/HLA-A2 with high affinity</td>
</tr>
<tr>
<td>A6-X15</td>
<td>Engineered human scTv that recognizes Tax/HLA-A2 with high affinity</td>
</tr>
<tr>
<td>ACT</td>
<td>Adoptive cell transfer</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AGA-2</td>
<td>A-agglutinin adhesion subunit; Yeast mating protein</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin fluorophore</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>β2M</td>
<td>Beta-2-microglobulin</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor; membrane bound antibody</td>
</tr>
<tr>
<td>BiTe</td>
<td>Bispecific T-cell engager</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Cα</td>
<td>Constant region of the TCR-alpha chain</td>
</tr>
<tr>
<td>Cβ</td>
<td>Constant region of the TCR-beta chain</td>
</tr>
<tr>
<td>C&lt;sub&gt;H&lt;/sub&gt;</td>
<td>Constant region of the antibody heavy chain</td>
</tr>
<tr>
<td>C&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Constant region of the antibody light chain</td>
</tr>
<tr>
<td>CAR</td>
<td>Chimeric antigen receptor</td>
</tr>
<tr>
<td>CD3</td>
<td>Cluster of differentiation 3; T cell co-receptor</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of differentiation 4; T&lt;sub&gt;H&lt;/sub&gt; co-receptor</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster of differentiation 8; CTL co-receptor</td>
</tr>
<tr>
<td>CD19</td>
<td>Cluster of differentiation 19; B-lymphocyte antigen</td>
</tr>
<tr>
<td>CD28</td>
<td>Cluster of differentiation 28; T cell co-stimulatory molecule</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>C-myc</td>
<td>Epitope tag derived from c-myc gene product</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte; also called T&lt;sub&gt;C&lt;/sub&gt;</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen 4</td>
</tr>
<tr>
<td>DARPin</td>
<td>Designed ankyrin repeat protein</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMF4</td>
<td>MART1-specific TCR used in melanoma therapy trials</td>
</tr>
<tr>
<td>DMF5</td>
<td>Avidity-engineered MART1-specific TCR used in melanoma trials</td>
</tr>
<tr>
<td>Eby100</td>
<td>S. cerevisiae strain use for yeast display</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half-maximal effective concentration</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment antigen binding of an antibody</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallizable of an antibody</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate fluorophore</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenlymethoxy carbonyl chloride</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Forkhead box P3; transcription factor</td>
</tr>
<tr>
<td>gp100</td>
<td>Human HLA-A2-restricted peptide with sequence ITDQVPFSV</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft versus host disease</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin; epitope tag</td>
</tr>
<tr>
<td>HER-2</td>
<td>Receptor tyrosine-protein kinase erbB-2</td>
</tr>
<tr>
<td>HBV Env</td>
<td>Human HLA-A2-restricted peptide against hepatitis B virus envelope protein with sequence FLLTRILT</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen; MHC is humans</td>
</tr>
<tr>
<td>HLA-A2</td>
<td>Human leukocyte antigen of the HLA-A*02 allele group; most prevalent human MHC allele</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HTLV</td>
<td>Human T cell lymphotrophic virus</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INF</td>
<td>Interferon</td>
</tr>
<tr>
<td>IMGT</td>
<td>International ImMunoGeneTics information system®; <a href="http://www.imgt.org">http://www.imgt.org</a></td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine activation motif</td>
</tr>
<tr>
<td>K&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Murine MHC allele K of b</td>
</tr>
<tr>
<td>L&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Murine MHC allele L of d</td>
</tr>
<tr>
<td>L</td>
<td>Flexible linker; typically sequence GSADDAKKDAAKKDGS</td>
</tr>
<tr>
<td>m6</td>
<td>Engineered scTv of the 2C TCR that recognizes QL9/L&lt;sup&gt;d&lt;/sup&gt; with high affinity</td>
</tr>
<tr>
<td>m33</td>
<td>Engineered scTv of the 2C TCR that recognizes SIY/K&lt;sup&gt;b&lt;/sup&gt; with high affinity</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic bead activated cell sorting</td>
</tr>
<tr>
<td>MART1</td>
<td>Melanoma antigen, Melan-A; Anchor modified human HLA-A2-restricted peptide with sequence ELAGIGILTV unless specified</td>
</tr>
<tr>
<td>Mel5</td>
<td>Human TCR that recognizes MART1/HLA-A2</td>
</tr>
<tr>
<td>MD</td>
<td>Molecular dynamics</td>
</tr>
<tr>
<td>MDM2</td>
<td>Human HLA-A2-restricted peptide with sequence VLFYLGQY</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NHL</td>
<td>Non-Hodgkin’s Lymphoma</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NK Cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NPT</td>
<td>Condition where moles (N), pressure (P) and temperature (T) are conserved in MD simulations</td>
</tr>
<tr>
<td>NTA</td>
<td>Nitrilotriacetic acid</td>
</tr>
<tr>
<td>NVT</td>
<td>Condition where moles (N), volume (V) and temperature (T) are conserved in MD simulations</td>
</tr>
<tr>
<td>NYESO</td>
<td>Human HLA-A2-restricted peptide with sequence SLLMWITQC</td>
</tr>
<tr>
<td>NYESO-Val</td>
<td>Modified human HLA-A2-restricted peptide with sequence SLLMWITNV</td>
</tr>
<tr>
<td>OX40</td>
<td>Secondary co-stimulatory molecule; Also known as CD128</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PAP</td>
<td>Prostatic acid phosphatase</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocyte</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD1</td>
<td>Programmed cell death protein 1</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin fluorophore</td>
</tr>
<tr>
<td>Pep/MHC</td>
<td>Peptide in complex with MHC molecule</td>
</tr>
<tr>
<td>PPI</td>
<td>Human HLA-A2-restricted peptide from preproinsulin with sequence ALWGPDPAAA</td>
</tr>
<tr>
<td>R1</td>
<td>Human TCR that recognizes MART1/HLA-A2</td>
</tr>
<tr>
<td>RD</td>
<td>‘Rational design’ yeast display library based on A6 scTv</td>
</tr>
<tr>
<td>RD1-MART1</td>
<td>MART1-specific scTv clone isolated from RD1 library</td>
</tr>
<tr>
<td>RD1-MART1^{HIGH}</td>
<td>High affinity MART1-specific scTv clone isolated from RD1-MART1 affinity maturation libraries</td>
</tr>
<tr>
<td>RD1-Tax-1</td>
<td>Tax-specific scTv clone isolated from RD1 library; identical protein sequence of A6-X15</td>
</tr>
<tr>
<td>RD2-MART1</td>
<td>MART1-specific scTv clone isolated from RD2 library</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root-mean-square deviation</td>
</tr>
<tr>
<td>RU</td>
<td>Response units</td>
</tr>
<tr>
<td>SA</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>SASA</td>
<td>Solvent accessible surface area</td>
</tr>
<tr>
<td>scFv</td>
<td>Single-chain fragment variable</td>
</tr>
<tr>
<td>scTCR</td>
<td>Single-chain T cell receptor; also called scTv</td>
</tr>
<tr>
<td>scTv</td>
<td>Single-chain T cell variable fragment; also called scTCR</td>
</tr>
<tr>
<td>SEREX</td>
<td>Serological analysis of recombinant cDNA expression libraries</td>
</tr>
<tr>
<td>SERPA</td>
<td>Serological proteome analysis</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>SIY</td>
<td>Murine K(^{b})-restricted peptide with sequence SIYRYYGL</td>
</tr>
<tr>
<td>SL9/HIVgag</td>
<td>Human HLA-A2-restricted peptide with sequence SLYNTVATL</td>
</tr>
<tr>
<td>SOE</td>
<td>Splicing by overlap extension PCR</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>Surv</td>
<td>Human HLA-A2-restricted peptide from survivin with sequence LTLGEFLKL</td>
</tr>
<tr>
<td>Surv-T2M</td>
<td>Anchor-modified human HLA-A2-restricted peptide from survivin with sequence LMLGEFLKL</td>
</tr>
<tr>
<td>T1</td>
<td>Human TCR that recognizes MART1/HLA-A2</td>
</tr>
<tr>
<td>T1-S18</td>
<td>Engineered human scTv that recognizes MART1/HLA-A2 with improved stability</td>
</tr>
<tr>
<td>T1-S18.45</td>
<td>Engineered human scTv that recognizes MART1/HLA-A2 with high affinity</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumor-associated antigen</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>Tax</td>
<td>Human HLA-A2-restricted peptide from HTLV with sequence LLFGYPVYY</td>
</tr>
<tr>
<td>TC(_C)</td>
<td>Cytotoxic T cell; also called CTL</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tel1p</td>
<td>Human HLA-A2-restricted peptide with sequence MLWGYLQYV</td>
</tr>
<tr>
<td>T(_H)</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumor infiltrating lymphocyte</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>T(_{reg})</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TSA</td>
<td>Tumor-specific antigen</td>
</tr>
<tr>
<td>UVpep</td>
<td>UV-cleavable peptide with sequence KILGFVFJY</td>
</tr>
<tr>
<td>V(_{\alpha})</td>
<td>Variable region of the TCR-alpha chain</td>
</tr>
<tr>
<td>V(_{\beta})</td>
<td>Variable region of the TCR-beta chain</td>
</tr>
<tr>
<td>V(_{H})</td>
<td>Variable region of the antibody heavy chain</td>
</tr>
<tr>
<td>V(_{L})</td>
<td>Variable region of the antibody light chain</td>
</tr>
<tr>
<td>wt</td>
<td>Wild-type</td>
</tr>
<tr>
<td>WT1</td>
<td>Wilms’ Tumor Antigen 1; Human HLA-A2-restricted peptide with sequence RMFPNAPYL</td>
</tr>
<tr>
<td>WT1-D13</td>
<td>Engineered human scTv that recognizes WT1/HLA-A2 with improved stability</td>
</tr>
<tr>
<td>WT1-D15</td>
<td>Engineered human scTv that recognizes WT1/HLA-A2 with improved stability</td>
</tr>
<tr>
<td>WT1-D13.1</td>
<td>Engineered human scTv that recognizes WT1/HLA-A2 with improved affinity</td>
</tr>
<tr>
<td>WT1-D13.1.1</td>
<td>Engineered human scTv that recognizes WT1/HLA-A2 with high affinity</td>
</tr>
<tr>
<td>WT1-P20</td>
<td>Human TCR that recognizes WT1/HLA-A2</td>
</tr>
<tr>
<td>WT1-P22</td>
<td>Human TCR that recognizes WT1/HLA-A2</td>
</tr>
</tbody>
</table>
CHAPTER ONE

INTRODUCTION

Overview of the Immune System

The human immune system is highly evolved with the capability to control a variety of infectious diseases by distinguishing “non-self” molecules (derived from pathogens) from normal “self” tissues. Human immunity is a complex, multilayered system: As a first line of defense, the innate immune system works as a physical and chemical barrier to infection and has the ability to immediately target pathogens through the recognition of generic patterns found among broad groups of microorganisms. The second line of defense is the delayed but more comprehensive response of the adaptive immune system that leads to the specific targeting of pathogens through the generation and expansion of specific recognition molecules originating from a highly diverse pool. Additionally, adaptive immunity generates immunological “memory” of antigens it encounters through long-lived B and T cells that allow for a quick, effective response in the event of a future encounter. A summary of the innate and adaptive immune system is shown in Table 1.1.

Innate immunity

The innate immune system serves as a first line of defense against invading pathogens. Unlike the adaptive immune system that takes days to react to invading
pathogens, the innate immune system is constitutively present and immediately poised to deploy its defenses through the use of anatomical barriers and humoral and cellular components. First, the epithelial layers of the skin and mucosal and glandular tissues provide a physical barrier to block pathogens from entering the body. Additionally, chemical barriers at these surfaces include acids that lower the pH (e.g. like in sweat and gastric secretions), anti-microbial proteins and peptides (e.g. lysozyme and phospholipases in tears and saliva), and the normal bacterial flora of the skin that can block the colonization of pathogenic microorganisms.

In the event that an invading pathogen is able to evade the anatomical barriers and infect human tissue, humoral innate defenses can lead to acute inflammation and the recruitment of phagocytic cells. These humoral defenses include the complement system and antimicrobial enzymes and peptides. The complement system includes a group of serum proteins that, when activated, can function to lyse pathogens or facilitate their clearance. Antimicrobial enzymes, such as lysozyme, can act to destroy a variety of microorganisms often through the disruption of pathogen membranes.

The cellular components of the innate immune system include neutrophils, macrophages, and natural killer (NK) cells. Neutrophils are the first cells to migrate to the site of an infection and act quickly to engulf invading bacteria via phagocytosis. They also act through the generation of reactive oxygen and nitrogen species and through the expression of antimicrobial peptides. Macrophages are also responsible for phagocytosis of foreign pathogens and are able to generate reactive oxygen and nitrogen species. They are larger in size and persist longer in tissues than neutrophils,
continuing to engulf dead cells and cellular debris. Additionally, when activated, macrophages are responsible for the secretion of inflammation mediators including cytokines and complement proteins\(^3\). Finally, NK cells are responsible for destruction of transformed human cells (e.g. virally infected or cancerous) upon down-regulation of major histocompatibility complex (MHC) proteins\(^4\).

The responses of innate immune cells such as neutrophils and macrophages are triggered primarily through receptors that recognize pathogen-associated molecular patterns (PAMPs). PAMPs are molecular motifs that are unique to microbes and viruses and are not found in normal cells. The Toll-like receptor (TLR) family of proteins is a class of membrane-bound receptors that have evolved to recognize PAMPs. Each TLR is responsible for the recognition of a different subset of ligands that collectively are able to identify a variety of bacteria, viruses, fungi, and protozoa. Activation of TLRs leads to changes in gene expression that ultimately lead to the production of inflammatory cytokines, interferons, antimicrobial enzymes and peptides, and chemotactic factors\(^5,6\). Importantly, activation of TLRs present on dendritic cells (DCs) is required for their maturation and ability to activate both CD4\(^+\) and CD8\(^+\) T cells in adaptive immunity\(^7\). Additionally, dendritic cells, and to some extent macrophages, serve as professional antigen presenting cells, bridging the innate and adaptive immune systems.

*Adaptive immunity*

Whereas the innate system recognizes generic patterns common among viruses and microorganism, the adaptive system has the ability to specifically recognize and
eliminate foreign antigens. The adaptive immune system developed in jawed vertebrates approximately 500 million years ago. The main feature of the adaptive system is the somatic development of clonally diverse B and T lymphocytes (B and T cells) each expressing a unique antigen receptor, allowing potentially any pathogen to be recognized in an antigen-specific manner. Upon recognition of a foreign antigen, lymphocytes become activated, undergo clonal expansion, and develop into mature effector cells, such as B or T cells.

**B lymphocytes and antibodies**

B cells express an antigen specific antibody on their membranes. Antibodies (also known as immunoglobulins) are glycoproteins consisting of two sets of identical polypeptide chains: heavy chains and light chains (Fig 1.1A). Each heavy chain is disulfide bonded to a light chain, and the two heavy chains are also covalently linked by additional disulfide bonds. At the amino terminal end of each heavy and light chain there is a single domain called the variable region that together form the antigen-binding sites responsible for antigen recognition.

The antibody repertoire is highly diverse and estimated to be over $10^{10}$ in size. The astonishing diversity of antibodies is accomplished through six mechanisms: (1) the presence of numerous V, D, and J gene segments, (2) somatic rearrangements of germline V-J segments in immunoglobulin light chain and V-D-J segments in immunoglobulin heavy chain, (3) junctional flexibility at the hypervariable region CDR3, (4) P- and N-nucleotide addition, (5) association of heavy and light chains, and (6)
somatic hypermutation\textsuperscript{10,11}. Generation of a particular heavy and light chain pair to form an antibody occurs during B cell development. Each B cell expresses only a single type of antibody, but collectively all B cells in the body express a large repertoire of antibodies.

B cells develop in the bone marrow. Prior to encountering antigens for which their surface antibodies are specific, B cells are considered “naïve” or “resting.” If the B cell surface antibody is reactive with a “self” antigen in the bone marrow, it will be deleted or undergo receptor editing to produce a non-“self” reactive surface antibody (receptor). Once B cells migrate to the periphery, they can then bind antigens through their membrane bound antibodies (also called B cell receptors, or BCRs). If the antigen encountered in the periphery is a chronically expressed “self” antigen it had not encountered in the bone marrow, the B cell can become “anergic.” However, if an acutely expressed foreign antigen binds to the BCR, two primary events lead to activation of the B cell: (1) the interaction of the BCR and antigen induces a series of signal transduction pathways, and (2) the BCR with bound-antigen is internalized, degraded, and delivered to the surface as antigen-derived peptides of 15 to 24 amino acids in length bound to a major histocompatibility complex (MHC) class II molecule. CD4\textsuperscript{+} T helper (T\textsubscript{H}) cells then specifically recognize and bind to the surface-displayed peptide/MHC (pepMHC) molecules, secreting factors that further activate the B cell.

Upon activation, a small subset of B cells will immediately produce a soluble pentameric version of the membrane-bound antibody (BCR), called IgM, which is capable of providing some protection to the infected host. Other activated B cells will
migrate to germinal centers of lymph nodes where they undergo further modifications. These include (1) somatic hypermutation, which generates mutations in the V region genes leading to affinity maturation of the antibody\textsuperscript{12}, (2) class switching, which allows for different effector functions of the antibody through the use of different heavy chain constant regions after intrachromosomal deletion and recombination events\textsuperscript{13}, and (3) differentiation of the B cell into either memory B cells or plasma cells\textsuperscript{14,15}.

Memory B cells, like naïve B cells, express membrane-bound antibodies, but have a much longer lifespan and can respond very quickly in event of another encounter with an antigen for which it is specific\textsuperscript{16}. Notably, the generation and maintenance of memory B cell populations is the main goal of vaccinations and boosters\textsuperscript{17}. On the other hand, plasma cells secrete high numbers of soluble affinity-matured, class-switched antibodies towards the latter end of the immune response\textsuperscript{18}.

Secreted antibodies of different isotypes or classes (i.e. IgM, IgG, IgA, and IgE) have specific functions and operate at select locations. Whereas IgM antibodies are low affinity requiring avidity effects for binding (i.e. pentamers have 10 antigen binding sites), IgG and IgA antibodies are typically higher affinity and are able to function as monomers and dimers, respectively. IgG antibodies are the predominant class of antibodies in the blood and extracellular fluid and function by the neutralization and opsonization of pathogens and activation of the classical pathway of the complement system (Fig 1.1a). As IgA antibodies are weaker opsonins and activators of the complement system than IgG, they function primarily as dimers on epithelial surfaces where they primarily neutralize pathogens. IgE molecules are typically bound to Fc
receptors on mast cells which, when bound to antigen (allergen), are stimulated to release various mediators leading to an allergic reaction\textsuperscript{19}.

The various effector functions of different antibody classes are enabled through their fragment crystallizable (Fc) region. The Fc portion of the antibody is comprised of the C-terminal domains of the heavy constant regions, whereas the antigen-binding fragment (Fab) is comprised of the N-terminal variable and first constant region domain of the heavy chain and the entire light chain (Fig 1.1a). The Fc regions of antibodies function through binding to Fc receptors (FcRs) on the surface of effector cells. Binding of an Fc to an FcR leads to various signaling and functional events, including the destruction of the antibody-bound pathogen or toxin through receptor-mediated endocytosis and/or via granzyme release\textsuperscript{20}.

Due to the ability of antibodies to specifically bind antigens with very high affinities, their long serum half-lives, and their ability to mediate immune effector functions, antibodies have been a primary focus of protein engineering strategies for therapeutics since the mid-1990s\textsuperscript{21}. For example, in order to improve the binding properties of antibodies engineered single-chain variable fragments (scFv) of antibodies consisting of the variable region of each heavy and light chain connected by a short flexible peptide linker have been utilized to engineer antibodies for antigen specificity and affinity using phage and yeast display (discussed in a later section). The scFvs often retain specificity and affinity when converted to the full IgG framework\textsuperscript{22} (Fig 1.1a). Additionally, engineered Fc regions have been generated to improve or alter the effector functions of antibodies\textsuperscript{23,24}. Despite the success of antibody-based therapeutics,
generation of antibodies with specificity for MHC-restricted antigens (i.e. antigenic peptide fragments displayed within a MHC-molecule on the surface of cells), although possible, has proven much more challenging. This could in part be due to the fact that antibodies did not naturally evolve to recognize this class of antigens, and thus obtaining antibodies with peptide antigen specificity (rather than MHC specificity alone) may be difficult. In the next section T lymphocytes, which recognize and bind to MHC-restricted ligands through their membrane bound receptor, are described.

**T lymphocytes and T cell receptors**

Like naïve B cells, T cells display an antigen-specific molecule on their surface that recognizes foreign antigens (called the T cell receptor, or TCR). However, unlike the BCRs of B cells which undergo somatic hypermutation and class switching allowing for their secretion as high-affinity soluble antibodies, TCRs remain cell-bound and are activated through binding to MHC-restricted antigenic peptides on the surface of antigen presenting cells (APCs). TCRs are αβ or γδ heterodimers consisting of variable and constant regions, similar to antibodies. The majority (>90%) of circulating TCRs in the body are comprised αβ TCRs (Fig 1.1b). The role of the less prevalent γδ receptor is less clear but they are thought to be involved in the inflammatory responses in the skin and epithelial tissues, are a primary source of interleukin-17 (IL-17) in acute infection, and do not require MHC presentation.

Although TCRs do not undergo somatic hypermutation, αβ TCRs are still highly diverse with estimated repertoire of over $10^{14}$ in size. Diversity is contributed through
five main mechanisms: (1) the presence of multiple V, D, and J gene segments, (2) somatic rearrangements of the V-J regions in the TCRα chain and V-D-J in the TCRβ chain, (3) junctional flexibility at the hypervariable region CDR3, (4) P- and N- nucleotide addition, and (5) the association of α and β chains (Fig 1.2a). A more in-depth description of the TCR structure and the molecular basis on how it engages its pepMHC ligand is described in the next section.

T cells originate from hematopoietic stem cells in the bone marrow like B cells, but unlike B cells they migrate to the thymus (hence ‘T’ cells) to differentiate and to undergo tolerance that ensures they are not activated by “self” antigens. During thymic tolerance (also called central tolerance), developing T cells undergo two selection processes: (1) positive selection which allow survival of only those T cells whose TCRs bind at low affinity to self-peptide/MHC molecules, and (2) negative selection which deletes T cells whose TCRs react with too high an affinity with self-peptide/MHC molecules (Fig 1.2b). This process ensures that mature T cells will not become activated when presented with a “self” antigen but will have a high probability of being restricted by MHC30,31.

Prior to tolerance induction, thymocytes become positive for expression of both CD4 and CD8 receptors (CD4+CD8+) in addition to the αβ TCR. The CD4 co-receptor, through its extracellular D1 domain, recognizes the β2 domain of MHC class II, whereas the CD8 co-receptor, through its extracellular α domain, recognizes the α3 domain of MHC class I (Fig 1.1b and Figure 1.2b). Interactions during positive selection between either CD4 or CD8 and its corresponding MHC class, together with the interaction of the
TCR and self-peptide/MHC ligands, determines whether the T cell becomes a CD4+ (or T helper, T_H) or CD8+ (or cytotoxic, T_C or CTL) T cell, respectively. Prior to migrating from the thymus, cells lose expression of the other co-receptor such that they are “single positive” (CD4+CD8− or CD4−CD8+)32. A subset of the CD4+ T cell population also expresses Foxp3, a transcriptional regulator, during central tolerance giving rise to another class of T cells, called regulatory T cells (or T_regs). T_regs are responsible for negative immunomodulation in the periphery in order to limit effector responses and further establish immunological tolerance33.

Following central tolerance, naïve CD4+ and CD8+ T cells migrate out of the thymus and into circulation and upon stimulation with a foreign pepMHC antigen they become activated (or “primed”) leading to the differentiation of a series of potential effector cell types. Stimulation of CD4+ T cells can generate a series of T_H cells (e.g. T_H1, T_H2, T_H17, and T_FH) whereas stimulation of CD8+ cells generates cytotoxic T cells (CTLs or T_c). In order for the this process to occur, three signals are required: (1) specific binding of the CD4+ and CD8+ T cell’s TCR to peptide-bound class II or I MHC, respectively, on the APC (2) costimulation by binding of CD28 on the T cell to a B7 molecule on the APC34, and (3) the stimulation by various cytokines, such as IL-2, to regulate differentiation into various effector cells35. As with B cells, a “memory” population of T cells is also maintained in order to respond rapidly to future infections36.

A CD4+ T cell, upon receiving appropriate cytokine signals, will develop into a specific type of T_H cell, such as T_H1, T_H2, T_H17, and T_FH37. Each of these different
subpopulations is characterized by a specific program of transcription factors, which result in subtype-associated cytokine profiles.

On the other hand, CD8$^+$ T cells become cytotoxic T cells (CTLs) that lyse target infected cells or cancer cells upon recognition. CTLs are critical to defending the host against intercellular pathogens, such as viruses, since they are capable of destroying infected cells through the recognition of foreign protein degradation products presented in the context of a class I MHC on the surface of APCs (Fig 1.3). CD8$^+$ T cell activation can be facilitated by stimulation of an effector CD4$^+$ T cell that interacts with the same APC. However, they can also be activated directly by mature dendritic cells. Due to the high costimulatory capacity of dendritic cells in activating CTLs, dendritic cell vaccines have been pursued in immunotherapy strategies to activate naïve CD8$^+$ T cells against tumors (described in detail later in the “Cancer Immunotherapy” section)\textsuperscript{38}.

Signaling of T cells through the TCR that occurs upon pepMHC engagement initiates a cascade leading to Ca$^{2+}$ efflux and Ras activation (Fig 1.3). In the proximal signaling complex, binding of TCR to pepMHC leads to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) of associated CD3 molecules by CD4/8-associated p$^{\text{LCK}}$. The CD3 complex consists of three polypeptide dimers each containing ITAMs on their cytoplasmic tails: heterodimers $\gamma\epsilon$ and $\delta\epsilon$, and homodimer $\zeta\zeta$ (occasionally a $\zeta\eta$ heterodimer). ZAP-70 kinase is recruited upon phosphorylation of CDR3$\zeta$ chains and phosphorylates adaptor molecules, such as SLP-76 and LAT, which recruit components of Ca$^{2+}$-, PKC-, and RAS-mediated pathways leading to transcriptional regulation through global regulators, such as nuclear factor of
activated T cells (NFAT) and nuclear factor kappa B (NF-κB)\textsuperscript{35}. Transcriptional changes lead to the CTL’s expression of potent cytotoxins, such as perforin, granzyme, and granulysin. Perforin forms pores in the infected cell’s plasma membrane causing lysis and allowing pro-apoptotic serine proteases, called granzymes, and granulysin to enter and induce apoptosis.

Due to the potent effector capacity of T cells and their ability to recognize intracellular antigen through surface-displayed MHC molecules through TCRs, immunotherapy strategies have focused on the engineering of T cells and their TCRs to better recognize tumors. Additionally, because the TCR evolved to recognize MHC-restricted ligands, it provides a useful alternative to monoclonal antibodies (mAbs) as targeting molecules to deliver cytokines or drugs to cells displaying MHC-restricted viral, cancer, or autoimmune antigens. Our group has developed a single-chain TCR format called scTCR or scTv that is structurally analogous to a scFv (Fig 1.1d) for these purposes. The next section provides a detailed view of the molecular basis of CD8\textsuperscript{+} T cell recognition of peptide/class I MHC through the TCR.

**Molecular Basis of T Cell Recognition**

As indicated above, in order for a T cell to make it through central tolerance, TCR binding affinity must follow the goldilocks principle: falling into a narrow range of possible affinities such that the TCR binds to pepMHC with sufficient affinity to be positively selected, but not too strongly as to be deleted during negative selection. As a result of this selection process and the fact that TCRs do not undergo somatic
hypermutation like their antibody counterparts, the natural TCR affinities for a foreign pepMHC are typically low, i.e. 1-100 µM. In this section I summarize the molecular basis of TCR:pepMHC recognition and the current view of how the structural features of both MHC and TCR allow for the affinity and specificity requirements of TCR binding and activation.

*Structural view of the major histocompatibility complex (MHC)*

There are two primary classes of MHC molecules: class I and class II. Class II MHC typically presents peptides to CD4+ T_{H} cells and is located on the surface of professional APCs, such as B cells, dendritic cells, and macrophages. Class II MHC consists of two chains (α1β1) that form a peptide-binding groove composed of a seven-stranded β sheet floor and two flanking α helices. Class II MHC-restricted peptides are typically 15-24 amino acids and extend past the open ends of the MHC molecule. In contrast, class I MHC molecules present peptides to CD8+ CTLs and are found on the surface of all nucleated cells. The class I MHC molecule consists of two polypeptide chains: the heavy chain (composed of three domains, α1α2α3) and the light chain (β2-microglobulin or β2m)(Fig 1.4). The α1 and α2 domains form the peptide-binding groove of the MHC molecule, and the α3 domain non-covalently associates with β2m and anchors into the APC membrane. Despite composition by different chain lengths, the overall structural architecture of class I and II MHC are the same; however, the ends of the flanking α1 and α2 helices of class I MHC are closed and smaller (8-10 amino acid) peptides are bound and presented to CTLs.
The peptides presented in the context of the MHC class I molecule on the surface of APCs are derived from the products of proteolysis (Fig. 1.4b). Proteolysis products include those digested in the cytosol by the proteasome as well as those internalized from exogenous sources and digested by lysosomal degradation\(^4^1\). Peptides that are formed in the cytosol are typically translocated to the endoplasmic reticulum (ER) via an ATP-dependent transporter associated with antigen processing (TAP) and assembled with MHC class I heavy and light chains with aid from chaperone molecules, such as calnexin and tapasin. Although peptides derived from endocytic pathway are typically presented on class II MHC molecules, cross-presentation of exogenous antigens can occur such that endocytic degradation products are presented on class I MHC. Although the mechanism of cross-presentation is still unclear, it is thought it could occur through peptide exchange in the endosome or by the presence of endocytic peptides in the ER. In this regard, peptides presented in class I MHC represent the full protein repertoire of the cell, included intracellular, cell-surface, and secreted proteins including those that result from viral infection or transformation of human cells to a cancerous state. Additionally, it is notable that “self” peptide antigens presented on class I MHC molecules and polymorphisms in MHC itself often play a critical role in autoimmunity\(^4^2\).

In humans, the human leukocyte antigen (HLA) complex is the name of the locus that encodes products of the major histocompatibility complex (MHC). In mice, the locus is called H-2. Class I HLA expression occurs at three loci, A, B, and C, and the resultant gene products are HLA-A, HLA-B, and HLA-C. In mice there are two loci, K (H-2K) and
D (H-2D), and sometimes L (H-2L). HLA-A2 is the most prevalent human MHC allele, up to 50% in some populations, and is expressed in many ethnic populations\textsuperscript{43}. As a result, it is frequently used as a model MHC to study TCR:pepMHC interactions as well as a target for therapeutics.

*Structural view of the $\alpha\beta$ T cell receptor (TCR)*

Studies of model TCRs, particularly the mouse TCR 2C (which binds alloantigen QL9/L\textsuperscript{d}, self-antigen dEV8/K\textsuperscript{b}, and synthetic antigen SIYR/K\textsuperscript{b})\textsuperscript{44-46} and human TCR A6 (which binds to cognate antigens Tax/HLA-A2 and Tel1p/HLA-A2) have provided a wealth of information on TCR structure and function\textsuperscript{47,48}. T cell receptors (TCRs) are $\alpha\beta$ heterodimers consisting of a membrane-proximal constant regions and distal variable regions (Fig. 1.1b and Fig. 1.4). The TCR variable regions of both the $\alpha$ and $\beta$ chain form the antigen-binding site with six loops, called complementarity determining region (CDR) loops, that provide the binding energy with pepMHC\textsuperscript{40} (Fig 1.4b,c). Each chain ($\alpha$ and $\beta$) contains a CDR1, CDR2, and CDR3 loop: CDR1 and CDR2 loops are “germline derived” since they reside in the region encoded by each variable gene. As with antibodies, CDR3 loops are highly variable in sequence as they are encoded by the junctions of the somatically rearranged gene segments (Fig. 1.2a and Fig. 1.4b,c)

*TCR:pepMHC binding and activation*

Since the mid-1990s when the first crystal structures of two TCR:pepMHC complexes were solved\textsuperscript{49,50}, over two dozen additional structures have been reported.
These have collectively enabled extensive structural and biochemical characterization of the TCR:pepMHC interaction\textsuperscript{40}. These structural studies have shown that overall docking orientation and CDR loop positioning is similar in TCR:pepMHC complexes (Fig. 1.4b). This invariant orientation positions the germline-derived CDR1 and CDR2 loops of the TCR $\alpha$ and $\beta$ chains primarily over the MHC $\alpha_1$ and $\alpha_2$ helices, and the highly variable CDR3 loops of both chains primarily over the peptide. Although residues of CDR3 loops are thought to be primarily involved with making contacts with peptide residues whereas CDR1 and CDR2 residues are thought to primarily make contacts with MHC helices, there are cases where direct peptide contacts are made with non-CDR3 residues (particularly with CDR1$\alpha$) and CDR3 residues make contacts with MHC helices\textsuperscript{51}. Moreover, TCR specificity and engineering studies have suggested that up to all six loops are involved in specific recognition of the peptide, although they might not be involved directly in direct contacts. This phenomena has been attributed to two mechanisms: (1) “CDR editing,” in which the CDR3 loops of the TCR modulate interactions with germline derived CDR1 and CDR2 loops with pepMHC residues\textsuperscript{52}, and (2) “peptide editing,” in which TCR:pepMHC interactions are influenced by changes of the binding surface of the MHC depending on the peptide bound\textsuperscript{53}.

Although the molecular basis of the invariant TCR:pepMHC docking orientation is not fully understood, two prevailing models of TCR/MHC bias exist: (1) the germline codon bias model in which evolutionary pressure for TCR loop residues to yield a minimum basal affinity for MHC helices has shown a “preference” for productive contacts with MHC, eliminating TCRs which do not interact with MHC\textsuperscript{53,54}, and (2) the
co-receptor model in which the TCR:pepMHC interaction is “imposed” by the geometries required for productive interactions of the TCR complex and CD4/CD8 co-receptor during positive selection\textsuperscript{55,56}. Although compelling evidence for and against both models exists (including data presented in Chapter 2 of this dissertation\textsuperscript{57}), it is likely that both play an important role in the specificity of MHC restriction\textsuperscript{51,58}.

Where crystal structures provide insight on the likely endpoint of TCR:pepMHC, several models have been proposed for the binding mechanism, including a two-step binding mechanism and electrostatic steering\textsuperscript{40}. In order to distinguish foreign antigens from “self” in the periphery, TCRs must rapidly “scan” a very large number of pepMHC complexes. The two-step binding mechanism model suggests that the initial encounter of the TCR with pepMHC involves cursory interactions of primarily CDR1 and CDR2 TCR loops with the α1 and α2 helices of the MHC, followed by a more extensive sampling of the peptide by diverse CDR3 \textsuperscript{59}. On the other hand, the electrostatic steering mechanism suggests that long range electrostatic steering events between charged TCR and MHC residues pre-orient the complex for potential interactions\textsuperscript{40}. In any case, the structural changes of the TCR CDR loop positions that occur upon binding, as evidenced from crystal structures of bound and unbound TCRs, suggest that both highly dynamic and more subtle binding mechanisms are plausible and they involve mechanisms such as rigid-body association, induced fit binding, and conformational selection from a pre-existing equilibrium\textsuperscript{60}.

Additionally, despite thorough characterization of kinetic and thermodynamic properties of TCR binding, the precise requirements for T cell activation remain
elusive\textsuperscript{39,61}. Particularly, there has been considerable debate on what factor plays a
decisive factor in whether or not a T cell is activated upon engagement of its TCR with
pepMHC: affinity or off-rate. The importance of off-rates in T cell activation are evident
by two models of T cell activation that suggest there is an “optimal dwell time” in
TCR:pepMHC binding: (1) the kinetic proofreading model, in which TCR:pepMHC
interactions must be sufficiently long enough to allow time for intracellular signaling
events resulting in agonist activity\textsuperscript{62}, and (2) the serial triggering model, by which the off-
rate must not be too long as to allow multiple TCRs to bind to and by activated by a
single pepMHC molecule\textsuperscript{63}. Although off-rates play an important roll in T cell activation,
several studies indicate that on-rates are also important in determining agonist versus
antagonist activity in T cell activation as well\textsuperscript{64-66}.

Furthermore, although TCRs are typically thought to be highly specific in terms of
their immunological functions, it is clear from numerous studies that TCRs are inherently
cross-reactive, allowing for the recognition of a range of self and foreign antigens\textsuperscript{67-69}.
This cross-reactivity has been attributed by a variety of mechanisms, including induced
fit, differential docking, structural degeneracy, and antigen-dependent tuning of pepMHC
flexibility\textsuperscript{70}.

Where the natural affinities of TCRs typically fall within the range of 1-100 \textmu M,
enengineering TCRs for higher affinity, different specificities, and for altered binding
properties is of considerable interest. In particular, engineering TCRs such that they no
longer require co-receptor (i.e. CD4 or CD8) binding to the MHC complex for activation
to occur could be beneficial, for example in recruiting the activity of CD4\textsuperscript{+} T cells against
a class I antigen. Previous work in our lab has shown that engineered receptors in the 2C mouse TCR system with affinities ($K_D$ values) below a 1 µM threshold are able to activate T cells independent of the CD8 co-receptor$^{71}$. The ability to activate a T cell in a co-receptor independent manner allows for $T_H$ cell responses upon binding of a class I pepMHC by the TCR (described later in the “Cancer Immunotherapy” section)$^{72}$.

**Immunity and Cancer**

Despite the ability of the immune system to prevent a myriad of infectious diseases, the ability of cancer to evade such host defenses remains one of the primary challenges of modern medicine. Although cancer has declined by 20% over the last two decades due to advancing treatment, diagnostics, and preventative medicine, it remains one of the leading causes of death in the United States and other parts of the world, claiming one in four lives$^{73}$. Because cancer arises from human cells and often not known pathogens, the “self/non-self” paradigm is not strictly applicable. In this section I discuss cancer markers and immunotherapy approaches currently under development as they pertain to TCR- and antibody-based targeting strategies.

*Cancer antigens*

Despite challenges of the immune system’s ability to recognize and/or eliminate cancer, some patients can generate antibodies and T cells against their tumors. The tumor targets include proteins that are mutated, misfolded, aberrantly expressed, or improperly modified. As a result, the isolation of autoantibodies and cancer-reactive
tumor infiltrating lymphocytes (TIL) and the subsequent characterizations of the antigens they recognize has provided large number of therapeutic leads. These antigens, known as tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs), can now be routinely identified through the use of high throughput proteomics strategies, such as serological or T cell analysis of recombinant cDNA expression libraries (SEREX), serological proteome analysis (SERPA), and analysis of protein-based microarrays \(^7^4\).

As a result of these strategies, over 400 MHC-restricted cancer antigens have been identified and characterized (Fig. 1.5a) \(^7^5\). Furthermore, the National Cancer Institute (NCI) performed a pilot project for the “Acceleration of Translational Research” in 2009 that ranked many of these antigens according to a series of defined and weighted criteria (e.g. therapeutic function, immunogenicity, specificity, oncogenicity) as to their therapeutic potential \(^7^6\). Additionally, crystal structures of many of these complexes have been solved (Fig. 1.5b) \(^7^7\).

MHC-restricted cancer antigens fall into four broad categories \(^7^5\): (1) antigens resulting from mutated proteins, (2) shared tumor-specific antigens, (3) overexpression antigens, and (4) differentiation antigens (Fig 1.5). Certain antigens, such as antigens resulting from mutated proteins, are highly specific to cancer, as they are not found in any normal healthy tissues. In terms of the development of therapeutics, these antigens (TSAs) represent the ideal targets, as the risk of adverse effects is minimal in other tissues \(^7^8\). The major disadvantage in terms of the development of therapeutics is that it requires that each treatment to be individualized, as oncogenic mutations typically vary
from patient to patient. Another class of TSAs includes the shared tumor-specific antigens, named as they are found on many different tumors. These include the cancer/testis antigens (e.g. MAGE-A family members) that are considered to be cancer specific as they are typically only expressed in the placenta or in germ line cells (which do not express MHC class I), although adverse effects in recent clinical trials have demonstrated that expression can occur in somatic cells\textsuperscript{79}. Differentiation antigens are a type of tumor-associated antigens (TAAs) that are expressed in both cancer cells and in the cell lineage from where the malignancy developed (e.g. tyrosinase, gp100, MART-1). Finally, some proteins are overexpressed in cancers such that the resultant peptides can serve as overexpression antigens (e.g. Her-2, EGFR).

Because MHC restricted antigens are derived from the comprehensive protein repertoire (i.e. intracellular, cell-surface, and secreted proteins), it allows for virtually any protein to be targeted in cancer immunotherapeutic approaches through the recognition of a cell surface molecule (i.e. the pepMHC). To date, the success in engineering antibodies that specifically recognize pepMHC has been limited\textsuperscript{80-83}. However, the use of TCRs to target this class of antigens in cancer immunotherapies offers unique advantages: (1) TCRs evolved to recognize MHC-restricted peptide antigens and as a result, the docking orientation and CDR loop footprint is largely invariant; this facilitates the engineering of TCR-based targeting molecules by allowing focused mutagenesis on residues most likely to contact the antigenic peptide. (2) TCRs, like antibodies, have the capacity to generate huge numbers of unique antigen-specific binding molecules. Although affinities are low as a result of central tolerance and the fact that they do not
undergo somatic hypermutation like their antibody counterparts, TCRs can now be engineered \textit{in vitro} to very high affinities (nanomolar and picomolar range). (3) TCRs are composed of immunoglobulin (Ig) folds like antibodies and are amendable to similar engineering and therapeutic formats (e.g. Fc-receptor fusions).

The cancer antigens focused on in this dissertation are those that have met two general criteria: (1) restriction by HLA-A2, the most prevalent human MHC allele\textsuperscript{43}, and (2) availability of TCRs isolated from reactive T cells that use the highly stable V\textalpha{}2 (TRAV-12) gene segment which is amendable to yeast display engineering (described in the ‘TCR Engineering’ section). Two antigens I engineered high affinity TCRs against were MART-1/Melan-A (Chapters 2 and 4) and Wilm’s Tumor Antigen (WT1; Chapter 3)(Fig 1.5b).

MART-1 is a differentiation antigen that is expressed in over 80\% of metastatic melanomas\textsuperscript{84} as well as in melanocytes in the eye, ear and skin\textsuperscript{85,86}. In the 2009 NCI cancer antigen prioritization project, this antigen was ranked #14 of the top 75 antigens examined. MART-1 was one of the first MHC-restricted TAAs to be identified for the use as a therapeutic target of melanoma. Initial successful adoptive cell transfer (ACT) trials with melanoma TILs found that many of the T cell were reactive with MART-1/HLA-A2\textsuperscript{87}. As a result, several clinical trials involving the transfer of human T cells transduced with genes that encode a MART1-specific TCR were pursued. The first published study in 2006 involved the transfer of an allogeneic T cells with a MART-1 specific TCR into 15 patients in which one patient has a partial response\textsuperscript{88}. Also in 2006, the seminal study describing the first successful clinical trial with gene-modified T cells was published by
researchers at the National Cancer Institute in which 4 of 31 melanoma patients showed sustained objective responses following ACT of autologous peripheral blood lymphocytes (PBLs) modified with a MART-1 specific TCR (named DMF4)\textsuperscript{89,90}. Finally, a 2009 NCI study by the same group utilizing genetically modified PBLs expressing an avidity-enhanced TCR against MART1 (named DMF5) shower partial responses in six of 20 patients, although on-target, off-tumor toxicity was demonstrated against normal melanocytes in the skin, eye, and ear\textsuperscript{91}. Although adverse affects could be partially treated in this study through the use of local steroid administration, it suggested that the risk versus reward of the use of affinity or avidity enhanced TCRs, particularly against the MART-1 antigen, should be thoughtfully considered in future trials\textsuperscript{91}.

The other primary antigen discussed in this dissertation, WT1, is an overexpression antigen derived from a transcription factor involved in differentiation and cell proliferation as well as apoptosis\textsuperscript{92}. Although initially classified as a tumor suppressor, it is clear that WT1 can also serve as an oncogene\textsuperscript{93}. Overexpression of WT1 has been detected in six hematological malignancies (e.g. acute myeloid leukemia (AML), chronic myeloid leukemia (CML), multiple myeloma), and over 30 solid tumors (e.g. brain, breast, pancreatic)\textsuperscript{94}. Despite low expression in normal tissues, thus far there have not been reports of toxicity in humans or mice with WT1-targeted immunotherapies\textsuperscript{94}. As a result of these and other factors, WT1 topped the list of the 2009 NCI cancer antigen prioritization project\textsuperscript{76}. Throughout the last decade, numerous WT1 peptide vaccine trials have been used in early-phase clinical trials for both hematological malignancies and solid tumors, and in general they have been well
 tolerated and safe although response rates have been low\textsuperscript{94}. Additionally, two phase I/II clinical trials are currently underway utilizing T cells transduced with WT1-specific TCRs against various types of leukemia at both the University College in London and the Fred Hutchinson Cancer Research Center (FHCRC) at the University of Washington (http://www.clinicaltrials.gov, NCT01621724 and NCT01640301, respectively). Although no results have been reported on these two trials to date, a pilot study at FHCRC demonstrated that in a cohort of 4 patients introduction of donor WT1-specific CTLs pretreated with IL-21 prior to transfer led to substantial memory T cell development, no recurrence of cancer (since 30 months following infusion), and lack of symptoms of graft versus host disease (GVHD)\textsuperscript{95}.

\textit{Cancer immunotherapy}

With the discovery and identification of TSAs and TAAs, and the development of a more comprehensive understanding on the interplay between tumors, the immune system, and the tumor microenvironment, immunotherapies against cancers have reached their golden age\textsuperscript{96}. In fact, Science magazine named cancer immunotherapy involving T cells as the 2013 breakthrough of the year due to promising results in recent clinical trials with PD1-directed therapies and adoptive T cell therapies, shifting the paradigm on how cancer is treated\textsuperscript{97}. Unlike traditional chemotherapies, immunotherapy focuses on targeting the immune system to eradicate cancer through the use of two main strategies: increasing immunosurveillance and decreasing immunosuppression. Cancer immunotherapies currently in use and under development include monoclonal
antibodies and antibody-like molecules, cell-based therapies, cancer vaccines, and other anti-cancer agents such as immune adjuvants (summarized in Table 1.2)\(^{98}\).

The use of monoclonal antibodies (mAbs) for the treatment of cancer has been one of the most successful therapeutic strategies for the treatment of both hematologic malignancies and solid tumors. To date, the FDA and EU have approved over 20 therapeutic antibodies with many more still in various stages of clinical trials. Strategies using therapeutic mAbs have aimed to block TAA/TSA, activate antibody effector functions, block immune checkpoints, inhibit signal transduction, and/or target drug molecules. Several therapeutic mAbs target receptors on the surface of tumor cells, such as HER-2 (e.g. trastuzumab), EGFR (e.g. cetuximab), and CD20 (e.g. rituximab), where others target molecules expressed by immune cells that are involved in immune signaling and regulation\(^99\). Of recent importance, mAbs that target receptors that negatively regulate T cell activation, such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD1), have been particularly successful in the clinic and in combination therapies. Ipilimumab, an FDA-approved anti-CTLA-4 antibody sold by Bristol-Myers-Squibb demonstrated a significant improvement to overall survival in a phase III clinical trial for patients of previously treated, advanced melanoma\(^{100}\), and has shown even more promise when used in combination with an anti-PD-1 antibody. Use of an anti-PD1 antibody, nivolumab, with ipilimumab lead to a 40% objective response rate and demonstrated a manageable safety profile\(^{101}\).

Monoclonal antibodies have also been used as bispecific molecules by linking a TAA/TSA-targeting scFv to a second scFv that binds an invariant region of the TCR.
complex (i.e. CD3). Binding of these bispecific molecules results in the activation of T cells against the tumor cell expressing the TAA/TSA regardless of its TCR-mediated specificity\textsuperscript{102}. Blinatumomab, a CD19/CD3-bispecific antibody that has been used clinically for the treatment B cell malignancies, has shown significant promise over the use of the CD19-specific antibody alone in ongoing trials with high objective response in ALL and non-Hodgkin’s Lymphoma (NHL)\textsuperscript{102}. Other uses of antibodies include the development of antibody-drug conjugates\textsuperscript{103} and radio-immunoconjugates\textsuperscript{104}.

Another immunotherapy strategy involves cancer vaccination, whereby dendritic cells or adjuvants are pre-loaded with TAA/TSA through the use of peptide cocktails, DNA and RNA vectors encoding TAA/TSA, or through gene therapy strategies\textsuperscript{105}. In April 2010, the FDA approved the first cell-based cancer vaccine, Sipuleucel-T (commercially known as Provenge\textsuperscript{TM} and sold by the Seattle-based biotech company Dendreon), for use in patients with metastatic prostate cancer\textsuperscript{106}. In this therapy, autologous peripheral blood mononuclear cells are isolated from the patient and treated with a recombinant prostatic acid phosphatase (PAP) cocktail to load and mature APCs ex vivo, and are then reintroduced into the patient. PAP has been shown to be expressed in \textasciitilde 95\% of prostate cancers\textsuperscript{107}. In a phase III clinical trial, Sipuleucel-T treatment led to an extension of median survival of 4.1 months over placebo\textsuperscript{108}, although there has been significant criticism of the design of this trial, as tumor responses in patients have been limited since the drug hit the market\textsuperscript{109}.

Another category of targeted immunotherapies includes adoptive cell therapies, which include the use of autologous TILs or genetically engineered T cells expressing a
TAA/TSA-specific TCR or chimeric antigen receptor (CAR)(Fig. 1.6 and Fig. 1.7). Adoptive cell therapies aim to increase the numbers of cancer-specific T cells and memory cells, and enhance the anti-tumor response through the release of cytokines and or cytotoxins\textsuperscript{110}. Initial studies by Steven Rosenberg and colleagues at the NCI have demonstrated positive outcomes of harvesting TILs from tumors, expanding and activating them \textit{ex vivo}, and reintroducing them into the patient\textsuperscript{111} (Fig. 1.6; navy arrows). However, in many cases cancers are able to down regulate immune activity making it a challenge to isolate adequate numbers of TILs using this approach. As a result, strategies to reprogram T cells to recognize cancer have been employed through genetic engineering approaches\textsuperscript{112}.

The two main strategies used to redirect the activity of T cells include introduction of TCR or CAR genes (Fig 1.6; purple arrows). In the case of TCRs, the $\alpha$ and $\beta$ chain genes that form TCR heterodimers recognizing a TAA/TSA of interest can be introduced into a population of the patient’s polyclonal peripheral blood T cells to re-engineer their specificity (Fig. 1.6 and Fig. 1.7a). TCR genes introduced into T cells can be derived from several sources: (1) isolation from autologous T cell clones, (2) isolation from allogeneic T cell clones (that are not tolerized against the patients’ TAA/TSA), or (3) isolation from transgenic mice that express human HLA genes\textsuperscript{112}. In any case, the TCR genes isolated by these methods can be further engineered for enhanced affinity, avidity, or specificity using directed evolution and computational approaches (described in detail in the next section) or used without modification\textsuperscript{113}. One recent success using this strategy at NCI involved the autologous transfer of T cells expressing an NY-ESO-1
cancer/testis specific-TCR with IL-2 where 4/6 patients with synovial cell sarcomas showed objective responses (one patient has a partial response), and 5/11 patients with NY-ESO-positive melanomas showed objective responses (with two demonstrating complete remissions)\textsuperscript{114}.

Despite this success, the use of T cells with genetically modified TCRs presents several challenges. One challenge in this approach involves mispairing of α and β chains, as the autologous T cell population already expresses its own specific receptors\textsuperscript{115,116}. Mispairing of chains can be problematic if the native/transduced heterodimers produce TCRs that recognize “self” targets and lead to autoimmunity, although engineering strategies have been developed to favor pairing of the transduced chains\textsuperscript{110}. As mentioned in brief in the previous section in regards to targeting of TAA/TSAs, another challenge of targeting TAA/TSAs with gene-modified T cells is potential off- and on-target toxicity. Off-target toxicity occurs when the transduced TCR recognizes an unintended pepMHC (such as by recognition of structurally similar peptides or through mispairing) causing destruction of healthy tissue whereas on-target toxicity occurs when the transduced T cells recognize the intended target in a non-intended tissue. For example, two recent trials utilizing TCRs targeting the MAGE-A3 cancer testis antigen led in one case to cardiac toxicities through recognition of an structurally related peptide derived from titin in the heart (i.e. off-target toxicity)\textsuperscript{117,118}, and led in another case to neurological toxicities due to recognition of a similar epitope derived from the related antigen MAGE-A12 in the brain (i.e. off-target toxicity)\textsuperscript{79}. As a result of this and other studies where toxicities have been demonstrated\textsuperscript{91}, strategies to
assess antigen expression and cross-reactivity are at the forefront of adoptive cell transfer development\textsuperscript{110}. In particular, the roles of TCR affinity and cross-reactivity of affinity- and avidity-engineered TCRs are currently being investigated in both CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells as well as strategies to better evaluate the safety and efficacy of different TAA/TSA targets\textsuperscript{72}.

Another strategy for genetically engineering T cells with specificity involves the use of CARs (Fig 1.6 and Fig 1.7b). CARs act as artificial TCRs by combining the cytotoxicity and persistence properties of T cells with the specificity of mAbs. As with TCR-mediated adoptive cell therapies, CAR-mediated therapies involve isolation of T cells from a patient and genetic modification of the T cells ex vivo. However, rather than transduction of \(\alpha\beta\) TCR genes, CAR constructs typically utilize scFv genes fused to the signaling domains of a T cell molecule, leading to T cell activation by scFv (antibody) binding to a tumor cell surface antigen. First generation CARs described in the literature have been constructed by the fusion of an antibody-based binding fragment to ITAM-containing signal transduction domains, such as CD3\(\zeta\) or FcR\(\gamma\). Subsequent generations of CARs have included costimulatory signaling domains (e.g. CD28, 4-1BB, OX40)\textsuperscript{119}.

To date, CARs have been successful in trials targeting B cell malignancies based on recognition of CD19\textsuperscript{120-126}, which is only present on the surface of the B cell lineage. In a trial with a CD19-specific CAR with a 4-1BB cytoplasmic domain linked to CD3\(\zeta\), Carl June and colleagues at the University of Pennsylvania showed that two of three patients with advanced chronic lymphocytic leukemia (CLL) had complete sustained remissions and the third patient exhibited partial regression\textsuperscript{120,121}. In a separate study at
NCI, 6 of 8 patients with progressive B cell malignancies had objective remissions following treatment with anti-CD19 CAR T cells, combined with IL-2\textsuperscript{122}. The use of anti-CD19 CARs has been extended to use in acute lymphoblastic leukemia (ALL)\textsuperscript{126} and a variety of other cancers with many clinical trials underway\textsuperscript{127}.

Despite success with CAR-mediated adoptive cell therapy trials, several challenges remain in its development to a routine medical practice. Where CARs avoid the risk of mispairing that could occur with TCR-mediated adoptive transfers, toxicity has been observed. For example, toxicities associated with anti-CD19 CAR strategies have included B cell aplasia, tumor lysis syndrome, and cytokine release syndrome\textsuperscript{127}. Additionally, other on- and off-target toxicities are possible, especially as CAR-strategies are applied to non-CD19-directed therapies. As a result, strategies to regulate CAR expression and/or T cell survival to mitigate these risks are currently being pursued\textsuperscript{119}.

**Protein Engineering for Immunotherapy**

Because of the central role of the immune system in cancer, autoimmunity, and infectious disease, protein engineering strategies based on core immunological principles provide an opportunity to exploit the immune system for the treatment of many human diseases. The field of protein engineering emerged in the 1980s leading to the generation of the most rapidly expanding class of new drugs: protein-based therapeutics (also called “biologics”). A main focus of protein engineering strategies has been to modulate the affinity and specificity of proteins, such as mAbs, which can be
used in immunotherapy and diagnostic applications. Here I discuss the types of binding molecules currently being developed, the engineering strategies used to modulate binding properties (particularly directed evolution by yeast display), and finally the background of TCR engineering that lays the foundation for the work described in this dissertation.

**Antibody and TCR engineering formats**

Since the advent of hybridoma technology in 1975\textsuperscript{128}, monoclonal antibodies have become the primary framework for the development of specific binding molecules for the use in research, diagnostic, and therapeutic applications. Initial antibodies developed in the late 1980s as potential therapeutics were generated in mice, and as therapeutics they had significant drawbacks in terms of immunogenicity and minimal activation of Fc-mediated effector functions. As a result, chimeric mouse-human antibodies were developed in which the variable regions of murine antibodies were grafted on to the constant regions of a human antibody\textsuperscript{129}. In the early 1990s, the advent of phage display\textsuperscript{130}, an entirely *in vitro* technique by which the variable domains of antibodies could be evolved, allowed for the first fully human antibodies to be developed, reducing the immunogenicity for therapeutic applications. Since then, the use of a variety of antibody formats have been utilized, including scFv, Fab domains, and other multivalent fragments, for the specific binding of therapeutic targets (Fig. 1.1a,c)\textsuperscript{131}.
Whereas antibodies have been successful in targeting numerous cell surface and soluble antigens, targeting of MHC-restricted antigens with antibodies has had limited success\textsuperscript{88-83}. Because the TCR evolved to recognize the class of MHC-restricted antigens and like antibodies are naturally generated with substantial diversity, our group\textsuperscript{113} and others\textsuperscript{132} have proposed their use in both soluble and adoptive cell therapies. Although TCRs are naturally membrane bound, soluble single-chain TCR (scTCR or scTv) variants consisting of the extracellular TCR $\alpha$ and $\beta$ variable domains connected by a linker can be generated and used to engineer TCRs \textit{in vitro} in an analogous format to scFvs (e.g. V$\alpha$-linker-V$\beta$ or V$\beta$-linker-V$\alpha$)(Fig 1.1d and Fig. 1.4c)\textsuperscript{133}. In addition to TCRs, other binding scaffolds have been utilized in directed evolution approaches, such as designed ankyrin repeat proteins (DARPins)\textsuperscript{134} and fibronectin type III domain-based scaffolds (Adnectins\textsuperscript{TM})\textsuperscript{135}. In the next section I describe engineering approaches that have been used to alter the binding and specificity properties of TCRs, antibodies, and other binding proteins.

\textit{Engineering approaches}

Protein engineering strategies that are used in order to modulate the binding affinity and specificity properties of proteins include directed evolution\textsuperscript{136}, rational design\textsuperscript{137}, computational design\textsuperscript{138}, and combined approaches (e.g. “semi-rational design”)\textsuperscript{139}. The most successful strategy used to engineer antibodies and TCRs involves directed evolution through the use of display technologies, such as yeast display, phage display, mammalian cell display, bacterial display, and \textit{in vitro} display.
The general principle of these strategies involves the formation of a physical linkage of the protein or peptide being evolved to the gene that encodes it. Through genetic manipulation, large libraries of protein variants can be generated, from which variants with desired characteristics can be isolated, and the protein sequences can be determined.

The first display technology, phage display, was developed in 1985\textsuperscript{140} and since then a variety of additional display techniques with additional benefits have been developed. Phage display involves the targeting of the protein of interest to the phage surface through fusion to a viral coat protein. Typically, the M13 filamentous phage coat protein pIII is fused to the C-terminus of the protein being evolved, mutational libraries are made in the protein of interest (up to \(10^{10}\) in size), and the library is propagated in \(E.\ coli\). Virus particles can be isolated from culture supernatants, then selected for protein mutants with desired properties through a process called “panning.” This process involves repeated iterations of selections where the binding partner of the protein of interest is immobilized to the surface of a microtiter plate allowing virions with productive mutations to bind while virions with non-productive mutations do not. Following a series of washes of modulated stringency, the virus particles with productive mutations are used to re-infect \(E.\ coli\) allowing for variants with improved properties to be isolated after several cycles\textsuperscript{141}. Phage display has been used extensively for antibody engineering for both for affinity maturation of antibodies \textit{in vitro} and for the isolation of \textit{de novo} antibodies from synthetic libraries\textsuperscript{142}. 

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Since the advent of phage display, several competing technologies have been developed to address some of its limitations. *In vitro* display systems, such as ribosome, mRNA, and covalent DNA display, eliminate the library size limitations of phage and cell-based display methods as there are no transformation steps, and it also allows for recursive mutations; however, it does not allow for the post-translational processing of expressed proteins or the quality control checkpoints of the eukaryotic ER of other systems that is advantageous in the evolution of immunoglobulin-domain containing proteins\textsuperscript{143}. On the other hand, mammalian cell display allows for eukaryotic post translational processing including the complex mammalian glycosylation required for the full-length IgG frameworks, and it allows for tight control over selection parameters through the use of fluorescence activated cell sorting (FACs); however, it is rather limited as only small libraries can be generated ($10^3$ to $10^6$)\textsuperscript{144}.

Although different display techniques offer different advantages and limitations for various applications, yeast display, first published in 1997 by Boder and Wittrup\textsuperscript{145}, offers a good compromise for applications in antibody and TCR engineering. Yeast display involves the expression of the protein of interest on the yeast cell surface through a covalent linkage to the yeast cell surface (Fig 1.8). The protein of interest is typically cloned as a C-terminal fusion to the yeast mating protein AGA-2, which forms disulfide linkages to AGA-1 yeast mating protein on the cell surface upon being transported to the cell surface via the yeast secretory pathway. Typically, 10,000 to 100,000 copies of the protein of interest are expressed on each yeast cell, allowing for avidity effects that enhance selections\textsuperscript{145,146}. The advantages of yeast display include
the following: (1) eukaryotic post-translational processing including disulfide isomerization and glycosylation, (2) the quality control mechanisms of the yeast secretory pathway\textsuperscript{147-149}, (3) precise control over selections based on affinities and off-rates through the use of FACS\textsuperscript{150}, (4) the ability to produce relatively large libraries ($10^9$-$10^{10}$)\textsuperscript{151,152}, and (5) the ability to assess the evolved characteristics directly on the surface of yeast without the need to express large quantities of protein\textsuperscript{153-155}. Although yeast display has been used for antibody engineering\textsuperscript{156} and other protein engineering applications\textsuperscript{157}, our group has developed yeast display technology for the purpose of TCR engineering (summary of scTCRs engineered to date described in the next section)\textsuperscript{113}.

The general process for engineering scTCRs by yeast display involves the isolation of reactive T cells clone for the antigen of interest, cloning of the TCR genes, and generation of a single-chain construct from the full length $\alpha$ and $\beta$ chains ($V_\alpha$-linker-$V_\beta$ or $V_\beta$-linker-$V_\alpha$)(Fig. 1.9). The single-chain construct is then cloned into the galactose-inducible pCT302 yeast display vector, which expresses the scTCR as a C-terminal fusion protein to AGA-2 yeast mating protein. The vector also contains an N-terminal HA epitope tag between AGA-2 and the scTCR gene that is used to probe for expression, and typically a c-myc epitope tag is added to the C-terminus of the scTCR for monitoring expression of the full-length fusion protein. Because scTCR fragments lack TCR constant regions that stabilize the TCR on the surface of the T cell, scTCR constructs typically require mutations to generate a stabilized form to express on the surface of yeast\textsuperscript{113}. 


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In order to select for stabilized scTCR variants, mutations are introduced throughout the scTCR construct via error-prone mutagenesis and the variants are introduced into yeast cells. Following induction of scTCR expression, the yeast population is stained with a conformational-specific antibody that binds to epitopes on the Vβ and/or Vα chain, and the cells that express the most stable mutants are isolated by magnetic bead activated cell sorting (MACS) or FACS. Additionally, selections can be performed with antibodies against the c-myc epitope tag in order to eliminate scTCR library variants that result from truncations through introduction of pre-mature stop codons. Following various selection cycles, individual yeast clones expressing a single scTCR variant can be screened for desired properties directly on the yeast cell surface. The resultant stabilized scTCR mutant can then be used as a template for affinity maturation of the TCR by generation site-directed libraries in CDR loops (Fig 1.10).

Affinity maturation libraries, generally in CDR3 loops but occasionally in CDR1 and CDR2 loops, can be stained with soluble pepMHC reagents of various valencies and yeast displaying scTCR mutants with improved binding characteristics can be isolated in an iterative selection process with MACS and/or FACS as before. In general, initial selections identify mutants that bind to the pepMHC of interest and through each subsequent selection iteration, the stringency of selection is increased (e.g. through decreasing staining concentration of pepMHC, decreasing valency of staining reagents, or off-rate based selections). Following isolation and screening of individual variants on the surface of yeast, the scTCR gene can be introduced into a pET expression vector, expressed at high levels in E. coli, and refolded from inclusion bodies to produce a
soluble scTCR. Alternatively, the scTCR gene can be cloned into a variety of formats (e.g. full length TCR, CAR, bispecific) for assessment in therapeutic or diagnostic models.

_Engineering of T cell receptors_

For over 15 years, our group has developed strategies to engineer stable, high-affinity TCRs using yeast display of TCRs in a single-chain format (scTCR or scTv) that consists of the variable regions of the α and β chains connected by a flexible linker (Vα-linker-Vβ or Vβ-linker-Vα)\(^{113}\). Because normal TCR affinities are low (K\(_D\) = 10-100 µM), engineering for improved affinity, stability, and specificity properties allows for their potential use in research, therapeutic, and diagnostic applications. In particular, engineering TCRs to affinities of less than 1 µM allows for use in applications that allow for T cell activation independent of CD8 co-receptor (e.g. activation of TCR-modified CD4\(^+\) cells upon recognition of class I pepMHC complexes)\(^{71}\)

Early work in the lab focused on the engineering and characterization of the mouse TCR 2C, which recognizes alloantigen QL9/L\(^d\) and synthetic antigen SIYR/K\(^b\) with wild type affinities in the µM range, for stability and high affinity against its various ligands. The resultant high affinity scTCRs (m6 and m33) recognized their respective ligands QL9/L\(^d\) and SIYR/K\(^b\) with binding constants (K\(_D\)) of 10 nM and 30 nM, respectively\(^{158-160}\). Our lab also engineered 2C variants using a T cell display system, which allows for TCR selection in the native state\(^{161}\). In addition to engineering mouse TCRs which recognize class I pepMHC ligands, our group has also engineered the 3L2
TCR which recognizes the class II-restricted ligand Hb/I-E^K via yeast display to an affinity of $K_D = 50$ nM (from wild type $K_D = 16 \mu$M)$^{162}$. Despite successful yeast display engineering of mouse TCRs, until recently engineering human scTCRs by yeast display had proven more challenging. However, a 2011 study by Aggen et al. determined certain conditions, such as the use of the highly stable human $V\alpha2$ region (IMGT, TRAV12 family) and the introduction of a serine substitution at the polymorphic residue 49 in $V\alpha2$, that enabled yeast display engineering of human scTCRs$^{163}$. In this study, two human $V\alpha2$-containing TCRs that recognize the HTLV antigen Tax/HLA-A2 and the HIV antigen SL9/HLA-A2 (A6 and 868, respectively) that were previously engineered for high affinity by phage display$^{164,165}$, were engineered for stability and expressed as scTvs$^{113}$. This platform has allowed for the engineering of several additional $V\alpha2$-containing human scTCRs described in this dissertation$^{57}$.

In addition to TCR engineering by yeast display, other groups have engineered TCRs of enhanced affinity and avidity using a variety of formats and directed evolution approaches$^{113}$. For example, phage display has been used to engineer full-length TCRs with very high affinities (nanomolar to picomolar range) against various class I pepMHC complexes$^{164-167}$. Additionally, computational approaches have been used to guide improvements in TCR affinity$^{168-171}$. The applications of engineered high affinity TCRs include uses in both soluble and adoptive cell therapies described earlier, as well as diagnostic and research applications (Fig. 1.11). As soluble therapeutics, scTCRs can be used as biologic
agents for the delivery of drugs or cytokines to TAA/TSAs or to viral or autoimmune antigens. Additionally, scTCRs could be used as targeting modules in bispecific agents, directing T cell-mediated immune responses to infected or cancerous cells. For adoptive therapies, engineered TCRs can be used in the full-length format or as scTCRs as CARs in order to redirect the T cell response to diseased tissues. As diagnostics, high affinity scTCRs could be used to determine TAA/TSA expression of tumors. Finally, high affinity scTCRs provide an opportunity in basic science to study the principles of TCR triggering of the requirements of MHC restriction.

**Overview of Dissertation**

This dissertation focuses on the engineering and characterization of high affinity human scTCRs for potential therapeutic, diagnostic, and research applications, as well as for use in studies to understand basic principles of MHC restriction. The engineering aspects of this dissertation have focused on two goals: (1) yeast display engineering\(^{163}\) for the affinity maturation of human TCRs isolated from T cell clones that recognize cancer antigens of interest (described in Chapters 2 and 3), and (2) the establishment of a platform by which high affinity TCRs with diverse specificities could be evolved entirely \textit{de novo} from a single scTCR scaffold without the need to isolate a reactive TCR clone (described in Chapter 4). The resultant panel of engineered scTCRs also enabled a study of the molecular basis of MHC-restriction through the use of site-directed mutagenesis studies of various CDR loop residues (Chapter 2). The experiments
performed during the course of this project have addressed three main aims, the results of which are presented in Chapters 2-4 of this dissertation:

**Aim 1: Engineering of high affinity scTCRs against two defined cancer antigens**

To date I have engineered high affinity scTCRs against the MART-1/Melan-A$^{57}$ and WT1 antigens, recently ranked in a National Cancer Institute pilot project as the 14th and 1st priority cancer antigen therapeutic targets respectively$^{76}$. Using yeast display, high affinity variants of MART-1 and WT1 scTCRs were isolated and showed binding affinities of 45 nM$^{57}$ and 330 nM, respectively, for cognate peptide/HLA-A2 complexes, as measured by surface plasmon resonance (SPR). Both scTCRs could be expressed at high levels in *E. coli*, and the soluble protein could detect HLA-A2-positive APCs pulsed with the respective specific peptides. The engineering of the MART-1-specific scTCR is described in Chapter 2, and the engineering of the WT1-specific scTCR is described in Chapter 3. These receptors are currently being studied in adoptive T cell models by our group.

**Aim 2: Analysis of predicted key residues of TCR:pepMHC recognition**

In addition to their potential clinically, high affinity scTCRs provide a unique opportunity to study the energetic impact of several key residues in CDR1 and CDR2 loops. Because the TCR binds to the pepMHC in an invariant diagonal docking orientation, it has been proposed that several evolutionary conserved residues in TCR variable regions evolved to contact the MHC. High affinity scTCRs enable the ability to
assess the contribution of these residues, as measuring even small decreases in binding affinity in their wild type counterparts would reduce binding such that measurements are often out of the detectable range achieved by conventional SPR techniques.

In order to test the hypothesis that evolutionarily conserved residues dictate TCR recognition of MHC helices, I used the MART-1/HLA-A2-specific scTCR engineered in Aim 1 and two other high-affinity TCRs that all contain the same Vα region and recognize the same MHC allele (HLA-A2), with different peptides and Vβ regions. By performing site-directed mutagenesis, I measured the changes in binding energy of putative conserved residues in CDR1 and CDR2 of the three Vα2 regions. The results in the five residues examined showed the importance of one key germline codon residue, CDR2α Y51, whereas the other positions varied in their relative contributions to binding in the panel of TCRs. Additionally, selections of single-position, yeast display libraries in two of the key positions of the MART-1/HLA-A2-specific TCRs showed a strong preference for the wild-type germline residues although structurally similar amino acids were tolerated at the binding interface and mediated MHC-restriction. As a result, we propose that where the CDR1α residue Y51 could account for significant proportion of the binding energy associated with MHC restriction and perhaps even positive selection, there is plasticity in the requirements of other positions in the CDR1α and CDR2α depending on the Vβ usage and peptide recognized. These results are presented in Chapter 2.
Aim 3: Generation of a strategy, based on semi-rational design, to rapidly isolate scTCRs specific for a wide array of peptides loaded on HLA-A2

Because recognition of pepMHC is mediated entirely by specific residues on CDR loops and the majority of TCRs adopt a similar Ig-fold and docking angle, I proposed that a stabilized scTCR could be used as a template for directing specificity and high affinity for a wide array of peptides displayed on HLA-A2. In this study I was able to accomplish the directed evolution of TCR variants with a change in binding specificity to a non-cognate pepMHC. Using the human TCR A6, whose cognate ligand is Tax/HLA-A2, as a template, computational approaches were used to guide the design of yeast displayed single-chain TCR libraries degenerate in CDR1α, CDR3α, and CDR3β loops. Selection with a non-cognate pepMHC, MART-1/HLA-A2, resulted in the isolation of mutants that bound specifically to MART-1 and no longer to the cognate ligand, Tax.

Mutational studies of yeast-displayed mutants and comparative molecular dynamics (SMD) simulations of modeled TCRs suggest that a single position, TCRβ98, could account for the mechanism of the specificity switch in one MART1-specific variant, although reverting the residue at this position to the wild type A6 residue is not sufficient to regain Tax binding. Furthermore, a scaffold variant isolated from an alternative scaffold library for MART1/HLA-A2 specificity is unaffected by mutation at TCRβ98 and is thought to be able to accomplish MART1-specificity through a different mechanism. One of these variants has been further affinity matured via directed evolution to bind the selecting, non-cognate pepMHC at low nanomolar affinities. These results are shown in
Chapter 4. This scaffold-library approach is currently being further developed in the lab as a high throughput strategy to isolate designer TCRs with high affinity against virtually any desired target antigen.
Figure 1.1 | Diagram of the native antibody (IgG) and TCR structure and single-chain engineering formats. (a) Schematic of an IgG antibody with relevant fragments indicated. The heavy chain (C\text{\text{H}}) constant and variable regions (V\text{\text{\text{H}}}) are colored in light blue and blue, respectively, and the light chain constant (C\text{\text{L}}) and variable regions (V\text{\text{\text{L}}}) are colored in pink and red, respectively, with the N and C terminal ends indicated. Interchain disulfide bonds are indicated by green lines, and the fragment crystallizable (Fc) and one of two antigen-binding fragments (Fabs) are labeled directly. The fragment variable (Fv) is also labeled directly, and the yellow stars indicate where antigen binding occurs. (b) Schematic of the TCR binding complex of an \(\alpha\beta\) TCR on the surface of a CD\text{\text{8}}^+ T cell binding to a class I pepMHC on the surface of an antigen presenting cell (APC). With analogy to the antibody structure, the \(\alpha\) chain constant region (C\alpha) is colored in pink and variable region (V\alpha) is colored in red, whereas the \(\beta\) chain constant region (C\beta) is colored in light blue and the variable region (V\beta) is colored in blue. The CD3 dimers (\(\varepsilon\gamma\), \(\varepsilon\delta\), and \(\zeta\zeta\)) are indicated in orange, and the co-receptor CD8 (\(\alpha\beta\) heterodimer) is colored in purple. The variable regions of the TCR that make up the binding interface with pepMHC are indicated in a dotted box. The MHC molecule is shown as three heavy chain domains (\(\alpha\)1, \(\alpha\)2, and \(\alpha\)3; light green) associated to the \(\beta\)2m light chain (green) with a bound peptide (yellow star). (c) Schematic of a single-chain Fv antibody format. Variable light (V\text{\text{\text{L}}}; red) and variable heavy (V\text{\text{H}}; blue) chains are connected by a flexible linker, and the yellow star indicates where antigen binding occurs. (d) Schematic of a single-chain TCR (scTCR or scTv). With analogy to the scFv, variable \(\alpha\) (V\alpha; red) and variable \(\beta\) (V\beta; blue) chains are connected by a flexible linker. The yellow star enveloped by the \(\alpha\)1 and \(\alpha\)1 MHC helices (light green) indicates where MHC-restricted antigen binding occurs.
Figure 1.2 | T cell tolerance and somatic gene rearrangement of TCR genes. (a) Simplified schematic of gene rearrangement of TCR α and β genes by RAG recombinase. The TCRα gene is rearranged by the combination of V-J segments (top), where the TCRβ gene is rearranged by the combination of V-D-J segments (bottom). The final mRNA transcripts that encode the TCR protein product as a result of the rearranged segments are also shown (middle). The approximate diversities of the TCR gene segments at the α and β loci from the IMGT database [www.imgt.org] are listed in the accompanying table. (b) Positive and negative selection of thymocytes. First, T cell precursors undergo TCR gene rearrangement and migrate from the bone marrow to the thymus. Next, immature thymocytes (CD4⁺CD8⁻) undergo positive selection, where T cells expressing TCRs that bind to class I or II MHC survive and those that do not are deleted. Then, surviving thymocytes that bind too strongly to self-MHC or self-pepMHC molecules are deleted during negative selection. The resultant mature thymocytes (CD4⁺ or CD8⁺) migrate to peripheral tissues where they can encounter and be activated by foreign antigens.
Figure 1.3 | Schematic of CTL-mediated signaling. General steps are numbered: (1) Upon TCR recognition of pepMHC, the formation of the TCR signaling complex is initiated, including association of CD3 dimers (orange) and CD8 co-receptor (purple). (2) The CD8-associated p56Lck kinase phosphorylates ITAMs on CDR3ζ chains. (3) Phosphorylated ITAMs recruit the ZAP-70 kinase (green) to the signaling complex. (4) ZAP-70 phosphorylates adaptor molecules, such as SLP-76 and LAT. (5) Adaptor molecules recruit components of the Ca2+-, PKC-, and RAS/RAC-mediated pathways which ultimately lead in transcriptional changes leading to activation, differentiation, and expression of CTL signaling and effector molecule components.
Figure 1.4 | Structure of a TCR:pepMHC complex. (a) An example of a TCR:pepMHC complex (Mel5:MART-1/HLA-A2; PDB: 3HG1). The variable and constant regions of the α-chain are colored in red, and those of the β-chain in blue. HLA-A2 (α1, α2, α3, and β2m) is shown in gray and the peptide (MART-1; ELAGIGILTV) is in black. (b) The TCR CDR footprint on pepMHC. CDR1 and CDR2 loops are positioned primarily over the MHC helices where CDR3 loops are positioned primarily over the peptide. A conserved diagonal docking orientation with the Vα region laying over the α2 MHC helix and the N-terminal end of the peptide, and the Vβ region laying over the α1 MHC helix and C-terminal end of the peptide is observed in virtually all TCR:pepMHC structures to date. (c) The variable regions of the Mel 5 TCR (from the Mel5:MART-1/HLA-A2 crystal structure; PDB: 3HG1) indicating the general format of a scTv.
**Figure 1.5 | MHC-restricted cancer peptide antigens.** (a) Table listing the number of cancer peptide antigens of the listed categories characterized to date. Source: Cancer peptide database [http://cancerimmunity.org/peptide/][75] (b) Overlay of peptides from crystal structures of cancer peptide antigens. The shared tumor-specific antigen NY-ESO-1 [PDB: 1S9W][172] is shown in magenta, differentiation antigen MART-1 [PDB: 1JF1][173] is shown in green, and overexpression antigen WT-1 [PDB: 3HPJ][174] is shown in blue. HLA-A2 helices of the MART-1/HL-A2 structure [PDB: 1JF1][173] are shown in gray.
Figure 1.6 | Overview of adoptive cell therapy. Schematic showing the two general routes for adoptive cell therapy. (1) Tumor invading lymphocytes can be harvested from the patient, expanded and activated \textit{ex vivo}, then reintroduced (navy arrows), or (2) peripheral blood lymphocytes can be isolated from the bloodstream, genetically modified to express TCRs (engineered or allogeneic) or CARs that recognize tumors, expanded and activated \textit{ex vivo}, then introduced into the patient (purple arrows).
**Figure 1.7 | Genetically engineered T cells for adoptive cell therapies.** (a) TCR-mediated adoptive cell therapy involves the viral transduction of the full length α and β TCR genes into autologous T cells and utilization of the native signaling components to form the TCR signaling complex (i.e. CD8, CD3, etc.). (b) CAR-mediated adoptive cell therapy involves the transduction of an antibody-based binding fragment (usually a scFv) fused to the signaling domains of the T cell. An example construct includes a costimulatory signaling domain (e.g. CD28, 4-1BB; green) and an ITAM-containing CD3ζ domain (orange) fused to the scFv via the CD8 hinge transmembrane domain (purple).
Figure 1.8 | Yeast display engineering. Diagram showing the relevant cloning region of the pCT302 yeast display vector for yeast display of a scTCR (bottom panel). The Vβ (blue) and Vα (red) are expressed as a single chain fusion protein connected by a flexible linker to the C-terminus of AGA-2 yeast mating protein. Flanking the N- and C-terminal end of the scTCR construct are fused HA and c-myc epitope tags to probe for expression. Libraries generated by mutagenesis of the indicated construct are introduced into yeast via homologous recombination. The resultant library consists of a population of yeast cells with each cell expressing 10,000-100,000 copies of a single scTCR variant on its cell surface (top panel). Yeast can be probed for expression utilizing fluorescent antibodies against the HA or c-myc epitope tags, for stability via antibodies that recognize conformational epitopes on the Vβ (blue) or Vα (red), or for affinity or off-rates via binding to a soluble fluorescent pepMHC ligand.
Figure 1.9 | General selection workflow for isolating a stable scTCR. Sample selection scheme for isolation of scTCR clones with improved stability through the use of antibodies specific for conformational epitopes or the c-myc epitope tag.
Figure 1.10 | General selection workflow for isolating a high affinity scTCR. Sample affinity maturation scheme for isolation of scTCR clones with improved binding to pepMHC through the use of fluorescently labeled or bead-conjugated pepMHC ligands.
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<th>Innate Immunity</th>
<th>Adaptive Immunity</th>
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<tbody>
<tr>
<td>Components</td>
<td>• Physical barriers (skin, mucosal membranes)</td>
<td>• Cellular components (B and T lymphocytes)</td>
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<td></td>
<td>• Chemical barriers (e.g. pH, lysozyme, phospholipases)</td>
<td>• Antigen-specific receptors (Antibodies and TCRs)</td>
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<td>• Humoral defenses (e.g. the complement system)</td>
<td>• Cell-mediated killing (CTLs)</td>
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<td></td>
<td>• Cellular components (e.g. neutrophils, macrophages, NK cells)</td>
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<tr>
<td>Response Time</td>
<td>• Constitutively present</td>
<td>• Activated upon antigen encounter</td>
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<td></td>
<td>• Responds in minutes to hours</td>
<td>• Responds in days to weeks</td>
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<tr>
<td>Specificity</td>
<td>• Very limited</td>
<td>• Highly diverse and adaptive</td>
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<tr>
<td></td>
<td>• Recognition of general patterns (i.e. PAMPS)</td>
<td>• Recognition of specific antigens</td>
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<tr>
<td>Memory</td>
<td>• No memory</td>
<td>• Immunological memory</td>
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<td></td>
<td>• Response to infection same in repeated exposures</td>
<td>• Response to same antigen is faster and more effective in subsequent exposures</td>
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<tr>
<td>Treatment</td>
<td>Types</td>
<td>Mechanisms of Action</td>
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<tr>
<td>Targeted soluble therapies</td>
<td>Monoclonal antibodies (mAbs) and antibody-like molecules in various</td>
<td>• Targeting of drugs, toxins, or radioactive particles</td>
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<td></td>
<td>formats (e.g. scFv, bispecifics)</td>
<td>• Immune checkpoint blockade</td>
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<td></td>
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<td>• TAA/TSA or growth factor neutralization</td>
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<td></td>
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<td>• Inhibition of signal transduction</td>
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<td>• Activation of antibody-mediated effector functions (e.g. antibody-dependent</td>
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<td>cellular cytotoxicity (ADCC), complement)</td>
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<td>• Activation of the cell mediated immune response (bispecifics)</td>
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<tr>
<td>Cancer vaccination</td>
<td>Dendritic cells loaded with TAA/TSA, peptide vaccines, gene therapy</td>
<td>• TAA/TSA-specific activation and enhancement of immune response</td>
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<tr>
<td></td>
<td></td>
<td>• Cancer prophylaxis</td>
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<tr>
<td>Adoptive cell therapy</td>
<td>Autologous or allogeneic transfer of TILs, TCR or CAR genetically</td>
<td>• Generation of a TAA/TSA-specific lymphocyte population</td>
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<td>engineered T cells</td>
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<td>• Establishment of immunological memory</td>
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<td>Non-specific therapies</td>
<td>Cytokines (e.g. IL-2, INF-α, GM-CSF), immunomodulatory chemotherapies</td>
<td>• Tumor growth control</td>
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<td>• Promotion of apoptosis</td>
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<td>• Immunomodulation and activation of immune cells</td>
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Table 1.2 | Summary of immunotherapy strategies. Examples of immunotherapy strategies currently being utilized for the treatment of cancer and their mechanisms of action.
References


CHAPTER TWO

HUMAN HIGH-AFFINITY T CELL RECEPTORS AGAINST MELAN-A/MART-1: ENGINEERING BY YEAST DISPLAY AND ANALYSIS OF THE CONTRIBUTION OF T CELL VARIABLE REGION RESIDUES TO BINDING

Abstract

One hypothesis accounting for major histocompatibility complex (MHC) restriction by T cell receptors (TCRs) holds that there are several evolutionary-conserved residues in TCR variable regions that contact MHC. While this “germline-codon” hypothesis is supported by various lines of evidence, it has been difficult to test. The difficulty stems in part from the fact that TCRs exhibit low affinities for peptide-MHC (pepMHC), thus limiting the range of binding energies that can be assigned to these key interactions using mutational analyses. To measure the magnitude of binding energies involved, here we used high-affinity TCRs engineered by mutagenesis of CDR3. The TCRs included a high-affinity, MART-1/HLA-A2-specific single-chain TCR and two other high-affinity TCRs that all contain the same Vα region and recognize the same MHC allele (HLA-A2), with different peptides and Vβ regions. Mutational analysis of residues in CDR1 and CDR2 of the three Vα2 regions showed the importance of the key “germline


Authors’ contribution statement: The INRi and INRi-T1 T cell clones were isolated in the laboratory of H.B., and TCR genes were cloned by D.S. under the guidance of W.U. Surface plasmon resonance was performed by K.H.P. and S.J.B. under the guidance of B.M.B. All other experiments were performed by S.N.S. under the guidance of D.M.K. Data interpretation, manuscript preparation, and manuscript editing were performed by all the authors.
codon” residue Y51. However, two other proposed key residues showed significant differences among the TCRs in their relative contributions to binding. With the use of single-position, yeast-display libraries in two of the key residues, MART-1/HLA-A2 selections also revealed strong preferences for wild-type “germline codon” residues, but several alternative residues could also accommodate binding and hence, MHC-restriction. Thus, although a single residue (Y51) could account for a proportion of the energy associated with positive selection (i.e. MHC-restriction), there is significant plasticity in requirements for particular side-chains in CDR1 and CDR2 and in their relative binding contributions among different TCRs.

**Introduction**

T cell-mediated recognition of a foreign peptide bound to a product of the major histocompatibility complex (MHC) occurs through the αβ T-cell receptor (TCR), in a process that is referred to as MHC-restriction. TCRs contain six complementarity-determining regions (CDRs), three (CDR1, CDR2, and CDR3) in each α and β chain. CDR1 and CDR2 loops are “germline derived” since they reside in the region encoded by each variable region gene, which do not undergo either somatic mutation or rearrangements. CDR3 loops are more variable in sequence as they are encoded by the junctions of somatically rearranged gene segments (VJ in the α locus and VDJ in the β locus), similar to antibodies.

In virtually all of the structures of TCR:pepMHC complexes, the CDR1 and CDR2 loops are found to dock over the helices of the MHC protein, whereas the CDR3 loops
are positioned over the peptide where they can contribute most importantly to the antigen specificity of the reaction\textsuperscript{1,2}. Despite these generalizations, there are cases where CDR1 residues are near the peptide and CDR3 residues appear to contact the MHC protein\textsuperscript{3-6}.

While there is conserved, diagonal docking of TCRs over pepMHC ligands, the complexities associated with the diversity of TCRs, peptides, and MHC proteins have made it difficult to reveal conserved chemistries of the interactions that could account for the process of MHC restriction. More recent evidence, where the structures of TCRs with the same V regions and the same MHC-restricting elements have been compared, has led to the “germline codon” hypothesis. In this hypothesis, several key residues in CDR1 and/or CDR2 interact invariably with residues from the MHC protein, suggesting that these TCR residues evolved to establish the biochemical basis of MHC-restriction. Results of mutagenesis of these residues have been consistent with their contribution to binding\textsuperscript{7-11}, or even in the process of positive selection\textsuperscript{12}. However, these studies have been unable to reveal the energetic importance of the residues among different TCRs. A recent study suggested that there is no absolute requirement for specific residues within the germline $\text{V}_\alpha$ or $\text{V}_\beta$ loops, as it was possible to modify these very significantly and still achieve MHC-restriction and positive selection in the thymus\textsuperscript{13}. Whether these modified TCRs retained the diagonal footprint characteristic of conventional TCRs remains to be seen. Another study showed that the CDR3 regions can significantly alter the germline-encoded interactions\textsuperscript{14}.
Studies with the mouse 2C system showed that two of the predicted germline codon residues in CDR2β (Y46 and Y48) were unable to bind the SIY/K\textsuperscript{b} antigen when the residues were changed to alanines\textsuperscript{7,15}. More recent work with a high-affinity mutant of the mouse 2C (named m33), generated in CDR3α, showed that the TCR docked in an identical way as the wild-type TCR, and that all of the CDR1 and CDR2 contacts were preserved\textsuperscript{16,17}. In addition, both the 2C TCR and the m33 TCR had the same amino acid preferences at Y46, using an approach based on T-cell libraries\textsuperscript{18}. To the best of our knowledge, there have not been studies with human TCR single-site mutants to understand the issue of CDR1 and CDR2 contributions to binding properties, in large part because wild-type affinities are already so low. This prompted us to more fully exploit the idea of using high-affinity TCRs to determine the requirements and binding energies associated with MHC restriction in a human system.

Recently, we described the engineering of two human single-chain TCRs, 868 and A6, with high-affinity for HLA-A2-restricted peptides HIV (SL9 or Gag) and Tax, respectively\textsuperscript{19}. This study showed that the Vα2 (IMGT: TRAV12) region was stable compared to other V regions, and that a polymorphism in framework residue 49 of Vα2 facilitated the display, engineering, and expression of soluble scTv (single-chain T cell variable) fragments in Escherichia coli. Using this as a platform for work with additional human TCRs, here we have engineered a third Vα2-containing scTv against the MART-1/Melan-A peptide restricted by HLA-A2; MART-1 is a human differentiation antigen expressed in more than 80% of metastatic melanoma tumors\textsuperscript{20}. Collectively, these three TCRs, with the same CDR1α and CDR2α, provided an opportunity to examine the
energetic impact of several key residues. In addition, there are several published crystal structures of Va2-containing TCR:peptide-HLA-A2 (pepHLA-A2) complexes, including the A6 TCR, providing a structural framework for the interpretation of the results.

With the use of a combination of alanine-scanning mutagenesis, and library-based selections with pepHLA-A2, our findings indicated (1) that two of the proposed CDR1 germline codon residues, R28 and Q31, contributed to binding in some cases, but not in others, consistent with recent structural studies suggesting that CDR3 editing can influence CDR1 contacts; (2) that one of the germline codon residues, Y51, exhibited similar and significant contributions to binding in all three cases (approximately 1.5-2 kcal/mol in free energy, ΔG); the free energy contributions of this residue, together with that contributed by one of the CDR1 residues, would be sufficient to produce an equilibrium binding constant (K_D value ~ 1 mM; ΔG ~ 4 kcal/mol) associated with co-receptor-dependent positive selection; and (3) that, using a library approach, the tyrosine at position 51 was preferred for pepHLA-A2 binding, relative to almost all other amino acids, although several conserved side chains (Phe and Trp) could be substituted. Thus, there is plasticity in the binding requirements of the germline-encoded residues, but the evidence suggest that there has been evolutionary pressure to maintain certain key residues.
Materials & Methods

Antibodies, pepHLA-A2, and flow cytometry

Antibodies used to detect yeast surface expression included the following: anti-HA epitope tag (Clone HA.11; Covance), anti-Vβ16 antibody (Clone TAMAYA1.2; Beckman-Coulter), anti-Vβ14 antibody (Clone CAS1.1.3; Beckman-Coulter), anti-Vα2 monoclonal antibody generated in our laboratory (data not shown), goat-anti-mouse IgG F(ab’)2 AlexaFluor 647 secondary antibody (Invitrogen), and streptavidin-phycoerythrin (SA-PE, BD Pharmingen). Peptides that bind to HLA-A2 [Tax11–19: LLFGYPVYV, SL977–85 (HIV-Gag): SLYNTVATL, MART-126–35A27L: ELAGIGILTV, MART-127–35: AAGIGILTV, and MART-127–35 A28L: ALGIGILTV] were synthesized by standard F-moc [N-(9-fluorenyl)methoxycarbonyl] chemistry at the Macromolecular Core Facility at Penn State University College of Medicine (Hershey, PA, USA). For FACS and flow cytometry analysis, recombinant soluble dimeric HLA-A2-Ig fusion protein (BD DimerX) was used. Additionally, a monomeric HLA-A2-biotin reagent generated by the exchange of a UV-cleavable peptide for another HLA-A2-restricted peptide in the presence of UV light was utilized to determine fold changes of binding in alanine mutants on the surface of yeast cells.22,23

Cloning and expression of scTv in yeast display vectors

TCR variable region fragments (scTv) were expressed in yeast display plasmid pCT302 (Vβ-L-Vα)24, which contains a galactose-inducible AGA2 fusion allowing for growth in Trp media.
Induction of the scTv gene involves growth of the transformed EBY100 yeast cells to stationary phase in selection media followed by transfer to galactose-containing media. The R1 and T1 single-chain genes for TCRs were synthesized by Genscript (Piscataway, NJ, USA) with an F49S mutation in the Vα2-domain of the construct

The MART-1-specific TCR genes were isolated from CTLs obtained by the MART-1/HLA-A2 multimer-guided cloning as previously described. The R1 and T1 scTvs consisted of the variable contains attached by the linker region GSADDAKKDAAKKDGS. The scTvs were introduced into the Nhel and Xhol restrictions sites of pCT302. The R1 scTv C105Fα mutation was introduced by site-directed mutagenesis using a Quikchange kit (Stratagene, La Jolla, CA, USA).

**Generation, display, and selection of mutated scTv yeast display libraries**

Error-prone PCR was used to generate random mutations, as previously described. CDR3 libraries were generated using splicing by overlap extension (SOE) PCR spanning 5 adjacent codons at a time (2 libraries in each CDR3 loop). Pre-SOE PCR products were generated for each of the four libraries utilizing the following primer pairs and the T1-S18 scTv as a template: β1, 5'- GGC AGC CCC ATA AAC ACA CAG TAT -3' (Splice 4L) and 5'- TGA AGA GGC GCA AAA ATA CAC ACC AGA ATC TTC CAG TTC GGC CGG TTG AAT TTT CAG GG -3', and 5'- GGA AGA TTC TGG GTA TTT TTG CGC CTC TTC ANN SNN SNN SNN SNN SGT TGA ACA GTA TTT TGG TCC AGG TAC CCG TC -3' and 5'- TAA TAC GAC TCA CTA TAG GG -3' (T7); β2, Splice 4L and 5'- TGA AGA GGC GCA AAA ATA CAC ACC AGA ATC TTC CAG.
TTC GGC CGG TTG AAT TTT CAG GG -3', and 5'- GGA AGA TTC TGG TGT GTA TTT
TTG CGC CTC TTC ACA TGC GGG TCT GNN SNN SNN SNN SNN STT TGG TCC
AGG TAC CCG TCT GAC C -3' and T7; α1, Splice 4L and 5'- CAC CGC GCA CAG ATA
AGT GGC TGA ATC AGA TGG TCG AGA ATC TCT AAT CAG CAG TGA AAC ATA
CTG AGA -3', and 5'- CCA TCT GAT TCA GCC ACT TAT CTG TGC GCG GTG NNS
NNS NNS NNS NNS CTG ATG TTT GGC GAT GGT ACC CAG CTG GTT GTG -3' and
T7; α2, Splice 4L and 5'- CAC CGC GCA CAG ATA AGT GGC TGA ATC AGA TGG
TCG AGA ATC TCT AAT CAG CAG TGA AAC ATA CTG AGA -3', and 5'- CCA TCT
GAT TCA GCC ACT TAT CTG TGC GCG GTG AAT GAT NNS NNS NNS NNS NNS
TTT GGC GAT GGT ACC CAG CTG GTT GTG -3' and T7. SOE PCR was performed
with each corresponding Pre-SOE along with both T7 and Splice 4L for each library (i.e.
β1, β2, α1, and α2).

Yeast libraries were made by homologous recombination in EBY100 yeast by
electroporation of error-prone or SOE PCR products along with NheI and XhoI digested
cDNA. The library was induced in galactose-containing media (SG-CAA) for 48
hours, washed with 1 ml of 1% PBS (phosphate buffered saline)/BSA (bovine serum
albumin), and stained with the following: anti-HA epitope tag (1:50), anti-Vα2 antibody
(1:50), anti-Vβ16 antibody (1:50) along with goat-anti-mouse IgG F(ab')2 AlexaFluor 647
secondary antibody (1:100), and corresponding pepHLA-A2 DimerX (100 nM) followed
by goat-anti-mouse IgG F(ab')2 AlexaFluor 647 secondary antibody (1:100). Cells were
washed (1 mL, 1% PBS/BSA), and the most fluorescent cells were selected using a
FACS Aria (BD Bioscience) high-speed sorter. Selection was performed on the error-
prone library for anti-Vβ16 antibody staining (1:50). In order to test thermal stability of isolated clones, we incubated yeast at elevated temperature for 30 minutes prior to the staining protocol. Each individual CDR library was sorted for positive Vβ16-staining, pooled in equal cell numbers, and expanded. The CDR3 library was then selected with MART-1/HLA-A2 dimer (10-100 nM).

Expression in E. coli, refolding, and biotinylation of soluble scTv fragments

T1 wild-type, T1-S18, and T1-S18.45 scTvs were introduced into the pET28a expression vector with a N-terminal 6-His tag using NcoI and EcoRI restriction sites (forward primer: 5’ TAT ACC ATG GGC AGC AGC CAT CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG CCG CGC GGC AGC GAA GCT GGT GTT ACT CAA TTC 3’, Reverse primer: 5’ T TTA GAA TTC TTA AAT ATT CGG TTT CAC AAC CAG 3’). Plasmids were transformed into the BL21 cell line, expanded, and induced for expression. Following induction, cells were passed through a microfluidizer (Microfluidics Corporation, Newton, MA, USA), inclusion bodies were isolated, and protein was purified as previously described\(^\text{30}\). Soluble scTvs were refolded and purified with Ni-NTA agarose resin (Qiagen, Valencia, CA, USA) followed by gel filtration (Superdex 200, GE Healthcare). Folded scTvs were biotinylated using N-hydroxysuccinimide (NHS) biotin ester (EZ-Link Sulfo-NHS-LC-Biotin Kit, Pierce/Thermo Scientific). Biotinylation was verified by gel-shift with streptavidin by SDS–PAGE (data not shown).
Binding of scTv proteins measured by SPR

The binding of purified refolded scTv proteins to cognate pepHLA-A2 was monitored with surface plasmon resonance (SPR) using a Biacore 3000 instrument. PepHLA-A2 complexes were generated by refolding from bacterially expressed heavy chain and β2m inclusion bodies as previously described\textsuperscript{31}. Due to the high affinities of the scTv proteins, a kinetic titration assay was utilized, in which increasing concentrations of analyte were sequentially injected over the surface without the requirement for disruptive regeneration injections\textsuperscript{32}. Experiments were performed with T1 wild-type (not detectable), T1-S18, and T1-S18.45 scTvs amine coupled to a standard CM5 sensor chip. PepMHC analyte was sequentially injected at various concentrations. The amount of immobilized scTv was kept below 500 RU and the flow rate was set to the maximum of 100 ml/min to minimize mass transport effects. Data were analyzed using Biaevaluation 4.1 as described previously\textsuperscript{32}. Solution conditions were 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM ethylenediaminetetraacetic acid, and 0.005% surfactant P-20, 25°C.

Binding of scTv fragments to peptide-pulsed antigen-presenting cells

HLA-A2\textsuperscript{+} human cell line, T2 was incubated at 37 °C for 2-3 hours with 1 μM MART-1 (ELAGIGILTV) or null SL9 (SLYNTVATL) peptide. Cells were then washed twice with 1% PBS/BSA, and incubated on ice for 1 hour with biotinylated scTv at various concentrations. Cells were washed twice with 1% PBS/BSA, followed by
incubation with SA-PE for 30-45 min on ice. Cells were washed twice and analyzed using an Accuri C6 Flow Cytometer. Experiments were performed with n=4.

**Alanine-scanning mutagenesis of scTvs**

Five site-directed alanine mutants (CDR1α D27A, R28A and Q31A, and CDR2α Y51A and S52A) were introduced into previously described high-affinity scTvs, A6-X15 and 868-Z11\(^{19}\), and T1-S18.45 by site-directed mutagenesis using a Quikchange kit (Stratagene, La Jolla, CA, USA). TCR-variable residue numbering has been kept consistent with sequence alignments with previously published high-affinity scTvs (Fig. 2.2 and Fig. 2.5)\(^{19}\). Yeast cells displaying the single-site mutants were titrated with cognate-peptide-exchanged HLA-A2 monomers in triplicate at 8 nM, 40 nM, 200 nM, 1 μM, and 5 μM and analyzed by flow cytometry\(^{22,23}\). Values were normalized using nonlinear regression analysis. Changes in binding affinity were approximated by determining the scTv concentrations at one-half maximal wild-type binding (5 μM). Independent experiments were performed with pepHLA-A2 dimers (BD DimerX) with similar results (data not shown).

**Yeast display library generation, selection, and 454 sequencing of scTv libraries**

Two libraries were generated in the high-affinity T1-S18.45 scTv construct at CDR1α residue Q31 and CDR2α residue Y51 using SOE PCR as described above. Each library was stained with 45 nM MART-1 (ELAGIGITV)/HLA-A2 and sorted via
FACS. Top staining (8-10%) clones were isolated, and amino acid sequences were determined by 454 sequencing (Roche/454 GS FLX+ Sequencer).

Results

Engineering a stabilized human scTv against MART-1/HLA-A2

The Vα2-positive MART-1-reactive T cell clones, INRi-T1 (referred to as T1; uses Vβ16) and INRi (referred to as R1; uses Vβ14), were isolated from melanoma-reactive T-cell lines. In order to engineer receptors with improved binding affinities, we cloned the V regions as a single-chain TCR fragments (scTvs) in the orientation Vβ-linker-Vα into a yeast-display vector. The scTvs were expressed on the surface of yeast via an N-terminal Aga2 fusion followed by a hemagglutinin (HA) epitope tag for detection of the displayed protein. Additionally, an F49S mutation was introduced into the Vα2-domain of the construct to confer thermal stability to the scTv, as described previously

Induced yeast cells containing the T1 scTv fusion (template)(Fig. 2.1a) were positive with an anti-HA epitope antibody (Fig. 2.1b). In order to detect individual Vα and Vβ domains, we used anti-Vα2 and anti-Vβ16 monoclonal antibodies for staining. Positive staining was seen with the anti-Vα2 antibody and weak staining was observed with the anti-Vβ16 antibody. Temperature stability assays have suggested that the Vβ16 antibody recognizes a conformational epitope whereas the Vα2 antibody recognizes a linear epitope (Fig. 2.4 and data not shown). This suggested that most of the T1 scTv fragments were not properly folded on the surface of yeast.
Induced yeast cells expressing the R1 scTv fusion on the surface were also positive for staining with an anti-HA epitope antibody; however, they did not show any detectable staining with the Vβ14 antibody (data not shown). Sequence alignments of the R1 TCR with the variable chain sequences of other Vα2-containing scTvs suggested that absence of the canonical J region “FGXG” motif (where X is any amino acid) at positions 105 to 108 of the TCRα, could potentially account for the poor stability of the R1 scTv on the yeast cell surface (Fig. 2.2a). As a result, the cysteine at position 105 of the TCRα was mutated to phenylalanine and stained at several concentrations of Vβ14 antibody; however, staining did not improve and only the T1 clone was pursued any further (Fig. 2.2b).

To generate a more stable T1 scTv that would be expressed on the surface of yeast, we performed random mutagenesis and selected for clones with improved surface expression by binding to the anti-Vβ16 antibody (Fig. 2.3). Previous work with mouse TCRs 2C and 3L.2 and human TCRs A6 and 868 have shown that selection of scTvs with enhanced yeast surface expression and temperature stability also correlate with increased soluble expression^{19,27,33}. Following two rounds of sorting, several mutants that had improved binding to the anti-Vβ16 antibody were isolated. Clones were screened for resistance to thermal denaturation using the Vβ16 as a probe (data not shown). One scTv mutant, called T1-S18, was highly temperature stable showing positive staining with the Vβ16 antibody when incubated at temperatures up to 80°C (Fig. 2.4). As a result, T1-S18 was selected for use as a template for affinity engineering (Fig. 2.1b, middle panels). The T1-S18 scTv was sequenced and shown to contain four
mutations: two in the Vα (S40P and Q80R), one in the Vβ (V80I), and one in the linker (K8E)(Fig. 2.5). Mutational analysis studies revealed that all four mutations (including that in the linker) contributed to the thermal stability of the T1-S18 clone (data not shown).

*Engineering of a high-affinity, surface-displayed T1 scTv fragment via site-directed mutagenesis*

Degenerate libraries were made in the CDR3 loops in order to select for mutants for increased affinity to peptide MART-1/HLA-A2-Ig dimers. CDR3 libraries of the T1-S18 template were sorted with two variants of the MART-1 peptide: the nonamer spanning residues 27–35 (AAGIGILTV)(Fig. 2.6a) and the decamer spanning residues 26–35 that contained a modified anchor residue at position 2 (ELAGIGILTV)(Fig. 2.6b). Some MART-1-specific TCRs have been shown to cross-react with nonamer, decamer, and anchor-modified decamer peptides\textsuperscript{34-37}. After four rounds of selection by FACS (fluorescent activated cell sorting) with both 9mer and 10mer MART-1/HLA-A2-Ig dimers, various clones were isolated and examined for binding. Clone T1-S18.45, isolated with MART-1 (ELAGiGILTV)/HLA-A2, showed significant binding, whereas the template T1-S18 and wild-type T1 did not (Fig. 2.1b). Clone T1-S18.45 also showed the greatest binding improvement to the 9mer variant of the MART-1 peptide (AAGIGILTV) on the surface of yeast, despite isolation with the anchor-modified 10mer peptide (Fig. 2.7). Sequencing revealed that T1-S18.45 contained five adjacent mutations in CDR3α, from wild-type NDNAR to SSSDF (Fig. 2.5).
Expression and binding studies of soluble T1 single-chain TCRs

The T1, T1-S18, and T1-S18.45 scTv genes (Vβ-linker-Vα) were cloned into an E. coli expression vector, induced to express recombinant scTvs, and refolded from inclusion bodies. Refolded preparations were purified by Ni-affinity and size-exclusion chromatography, yielding scTv proteins of the expected monomeric molecular mass of 30 kDa (Fig. 2.8a). Surface plasmon resonance (SPR) was performed with immobilized scTv fragments to determine kinetics and binding affinities for the various MART-1 peptide variants. Kinetic titrations were performed in order to avoid regeneration steps. SPR data analysis revealed nanomolar affinities of T1-S18.45 for all three MART-1 peptide variants examined (Fig. 2.8b and 2.8c). The stabilized T1-S18 variant bound with micromolar affinities, yielding affinity improvements of the T1-S18.45 scTv of 700-, 900- and 4,500-fold for the ELAGIGILTV, ALGIGILTV, and AAGIGILTV peptide complexes, respectively (Fig. 2.8c). The wild-type scTv T1 did not exhibit detectable binding, perhaps because it lacked adequate stability for immobilization and analysis. The micromolar affinities of the T1-S18, non-affinity-matured TCR are in the same range as typically seen for most pepMHC antigens.

To study whether the high-affinity, soluble scTv T1-S18.45 protein could detect pepHLA-A2 complexes on the surface of antigen-presenting cells (APCs), we biotinylated the protein utilizing a biotin-succinimidyl cross-linking agent\textsuperscript{38}. MART-1 (ELAGIGILTV) or null SL9 (SLYNTVATL) peptides were incubated with the TAP-deficient, HLA-A2\textsuperscript{+} human antigen-presenting cell line T2. Following peptide pulsing, we stained T2 cells with various concentrations of T1-S18.45-biotin followed by SA-PE, and
analyzed them by flow cytometry (Fig. 2.9). Specific staining for MART-1 was observed, yielding an estimated EC<sub>50</sub> affinity measurement of 120 nM, similar to that observed by SPR.

**Alanine-scanning mutagenesis of Vα2-containing high-affinity scTv fragments**

The availability of three Vα2<sup>+</sup> TCRs with high-affinity (A6-X15, 868-Z11 and T1-S18.45; called A6, 868, and T1 here, respectively), each for a different pepHLA-A2 ligand, allowed us to compare the exact binding contribution of proposed key CDR1 and CDR2 residues among different TCRs. To examine the binding energetics of these pepMHC interactions, we generated alanine mutants at five positions in a panel of high-affinity Vα2-containing scTv proteins: CDR1α residues D27, R28, and Q31, and CDR2α residues Y51 and S52. These residues have shown conservation based on TCR sequence alignments<sup>39</sup>, and the R28, Q31, Y51, and S52 side chains appear to be involved in contacts with the HLA-A2 helices in structures of three Vα2-containing TCRs (A6, Mel5, and DMF5; PDB files 1AO7, 3HG1, and 3QDG)(Fig. 2.10)<sup>3-5</sup>. Position D27 in CDR1α was the target of mutation for computationally-guided affinity increases in two Vα2-containing TCRs, A6 and DMF5<sup>40,41</sup>, and thus was also included in our analysis.

The yeast display system allows direct titrations of the fusion protein in order to examine affinities above a threshold K<sub>D</sub> of about 1 µM, thereby avoiding the need to purify each protein or mutant<sup>42</sup>. Accordingly, each alanine mutant was titrated with various concentrations of cognate pepHLA-A2 and analyzed by flow cytometry (Fig. 2.11a,b,c). To compare mutants, we calculated the magnitude of changes in binding
affinity from the concentrations of ligand that resulted in half-maximal binding of alanine mutant compared to “wild-type” high-affinity scTv (Fig. 2.11d). For all three scTv fragments, the tyrosine at position 51 was the only residue that uniformly contributed significant binding (10 to ≥15-fold reductions in binding affinity of the alanine mutants) to the pepHLA-A2 interaction, regardless of the Vβ or the peptide. Other positions showed minimal effects for all three TCRs (R28A and S52A), an effect on only one TCR (D27A in the A6 TCR), or an effect on two TCRs (Q31A in the A6 and 868 TCRs). Thus, these positions showed plasticity in their contribution to the binding energy.

In order to determine if the effect of the Y51A mutation observed in the high-affinity T1, A6, and 868 scTv fragments would hold true for the wild-type TCRs, we cloned variants of each scTv that contained the wild-type CDR loops, with or without the corresponding Y51A mutation, and expressed them in E. coli (note that the affinities of wild-type scTv fragments are too low to detect binding in the yeast display format). The 868 scTv fragment and its Y51A mutant were unstable and could not be used to make reliable SPR measurements. However, we were able to measure steady-state equilibrium binding parameters for wild-type T1 and A6 single-chain TCRs and their Y51A mutants (stabilized versions of these scTv, without the affinity mutations). These binding measurements yielded a ΔΔG value of over -2 kcal/mol for Y51A of the T1 scTCR (i.e., a K_D of 22 µM for the wild-type, whereas binding by the Y51A mutant could not be detected; hence, we estimated a K_D value of greater than 1 mM for the Y51A mutant). A ΔΔG value of -0.8 kcal/mol for Y51A of the A6 scTCR was measured (K_D values of 2.2 µM and 10 µM for wild-type and Y51A, respectively). Additionally, a recent
study by Piepenbrink et al. showed that the Y51A mutation in the wild-type, full-length A6 TCR yielded a ΔΔG value of -0.6 kcal/mol mol, similar to that of the Vα2-containing DMF5 TCR\textsuperscript{43}. Thus, we conclude that tyrosine 51 in the CDR2 of Vα2 contributes to binding energy among different TCRs in both the affinity-matured TCRs and the wild-type affinity TCRs.

\textit{PepHLA-A2 selections from single-residue libraries of the MART-1 TCR}

If during evolution the key CDR1 and CDR2 residues have been selected for MHC-binding, then pepMHC sorting of a yeast display library of all amino acids at these positions should yield enrichment for the evolutionary-driven residues. In order to determine the allowable residues at key positions of MHC restrictions, we generated degenerate libraries (NNS codons) at Vα positions 31 and 51 positions in the T1-S18.45 scTv yeast display vector. Each library underwent one round of selection with 45 nM MART-1/HLA-A2, and the top 8-10% binding clones were isolated by sorting. DNA from the enriched population of yeast was then subjected to 454 high-throughput sequencing to determine the amino acid frequency selected at each position (Fig. 2.12). At position 31, the wild-type glutamine was the highest frequency isolate, followed by alanine, cysteine, and valine (Fig. 2.12a). At position 51, tyrosine (wild-type), phenylalanine, and tryptophan were the highest frequency isolates (Fig. 2.12b). Thus, each positional library demonstrated a preference for the wild-type, energetically important residue, consistent with an evolutionary pressure to maintain these residues. However, several other amino acids were also capable of supporting pepMHC binding.
Discussion

Crystal structures of different TCR:pepMHC complexes have shown a conserved docking mode of TCR on the pepMHC such that the CDR1 and CDR2 loops are approximately positioned over the MHC helices and the CDR3 positioned over the peptide. Evidence for the co-evolution of these molecules has been shown by the presence of conserved residues and interactions in specific positions in CDR1 and CDR2 loops. In the current study, we focused on the energetic contribution of putative conserved TCR residues that shape immune recognition.

During development in the thymus, T cells are required to bind to MHC within a narrow affinity range in order to pass both positive and negative selection. Mutagenesis studies have shown that single residue differences in CDR1 and CDR2 loops are able to shift T cells out of this affinity such that negative selection or death by neglect can occur. Although the TCR’s intrinsic reactivity towards MHC could be partially masked by the negative selection process, structural and sequence data have suggested putative conserved residues that may play an important role in the binding energetics of the TCR:pepMHC interface.

Higher-affinity, single-chain TCRs provide an approach to rapidly assess alanine mutants using yeast display and flow cytometry without the need to express large quantities of protein as has been done in previous studies. Use of the higher-affinity scTvs allows for detection of up to 100-fold decreases in affinity that would not otherwise be measurable within the normal range for TCR:pepMHC (1-100 μM). Thus far, mutations that confer higher affinity on the variants of TCRs have most often been
engineered in the CDR3 loops, in order to retain peptide specificity\textsuperscript{19,27,46-51}. CDR1 loops often contact the MHC helices, and CDR2 loops tend to almost exclusively contact the MHC helices; thus, wild-type residues in these loops provide a good framework to access the binding energetics of “germline-encoded” contacts on HLA-A2 by alanine-scanning mutagenesis. Furthermore, the structures of several high-affinity mouse TCRs in CDR3 residues have shown virtually identical CDR1 and CDR2 contacts with MHC as the wild-type TCR\textsuperscript{16,17}. Thus, we believe that the high-affinity TCRs provide a useful surrogate for these wild-type, germline-encoded residues in CDR1 and CDR2.

Our data showed that the putative conserved CDR2\(\alpha\) residue Y51 contributed substantially to binding in all three of the high-affinity scT\(\alpha\) fragments examined. Although tyrosine at this position is not abundant (in 12-16\% of V\(\alpha\) regions), it interacts with the same region of MHC class I (near residue Q155) and class II (near B73) in various structures\textsuperscript{39}. Structural analysis of three V\(\alpha\)2-containing TCRs, A6\textsuperscript{5}, Mel5\textsuperscript{4}, and DMF5\textsuperscript{3}, suggest that the binding energy associated with mutation of Y51 to alanine could be due to the loss of contacts with HLA-A2 \(\alpha\)2 helix positions E154, Q155, and/or A158 (Fig. 2.10). In all three high-affinity scT\(\alpha\) fragments examined, mutation to alanine led to a 10- to \(\geq\)15-fold decrease in binding affinity (\(\geq\)1.4 kcal/mol in free energy).

While measurement of these interactions took advantage of the ability to use yeast display to rapidly analyze binding by the higher-affinity variants, we were able to confirm the energetic role of the tyrosine at position 51 in wild-type scT\(\alpha\) variants in which all CDR affinity mutations were reverted to the wild-type residues. These results are consistent with the similar docking and chemistries associated with wild-type TCR
and affinity-matured TCRs in the 2C system, showing only the CDR3 loop with mutations in a different position\textsuperscript{16,17}. Although qualitatively we show that Y51 contributed to binding in both high-affinity and wild-type TCRs, structural studies will be required to determine if there are changes in the docking of each of the loops and their residues. In this regard, a recent comparison of the A6 TCR:Tax/HLA-A2 complex with the high-affinity mutant A6-c134 TCR:Tax/HLA-A2 complex showed that the docking orientations were virtually identical\textsuperscript{21}. While there were subtle changes in the positions of the CDR3 and CDR2\(\beta\) loops, the positions of the CDR1\(\alpha\) and CDR2\(\alpha\), and in particular the position of the side chain of Y51, were very similar (Fig. 2.13). In their report, the authors proposed that most of the increase in binding affinity of the A6-c134 TCR was achieved through a greater number of interactions between the CDR3 loops and the peptide.

As positive selection is associated with weak affinities and free energies in the range of \(\sim 4\) kcal/mol\textsuperscript{52}, the contributions by this conserved tyrosine could reflect a substantial portion of this “minimal” affinity to ensure representation in the T cell repertoire. Early studies in the 2C system showed that affinities as low as 300 \(\mu\)M were able to yield agonist activity\textsuperscript{53}. Additionally we have shown that a single peptide point substitution, F5R, in the peptide of the 2C TCR:QL9/L\(d\) interaction reduced the affinity of the 2C TCR from 1.6 \(\mu\)M to 300 \(\mu\)M, yet the interaction was sufficient for agonist activity\textsuperscript{54}. Based on this, it is reasonable to assume that an affinity of 1 mM (\(\sim 4\) kcal mol\(^{-1}\)) or even lower (higher \(K_D\) value) is likely to be in the range for positive selection.

Although binding analysis showed a consistent role of CDR2\(\alpha\) residue Y51, more plasticity was observed in the contribution of CDR1\(\alpha\) residues. For example, the D27A
mutation led to a >15-fold decrease in binding of Tax/HLA-A2 by the A6-X15 TCR but this same mutation did not have a significant impact on binding by the other TCRs. The effect of the D27A mutation in the A6 TCR could be indirect, perhaps influencing the adjacent R28 through electrostatic interactions and affecting the stability of the entire loop. Similarly, the Q31A mutation caused a >15-fold change in both A6-X15 and T1-S18.45 TCRs, but the 868-Z11 TCR was not significantly affected. It has been proposed that CDR3 loops are able to modulate interactions of the MHC with germline-derived CDR1 and CDR2 loops in a process termed "CDR editing". This may account for the variation in binding contribution of residues in CDR1α. Analysis of contacts in crystal structure of Vα-containing TCRs DMF4, DMF5, A6, and Mel5 suggests a role of CDR1α in making peptide contacts in addition to MHC contacts, which may also explain differences in the energetic contribution with different peptide ligands (Fig. 2.10).

In order to further understand the requirements for binding, we generated single-codon, yeast display libraries at the two most energetically important CDR1 and CDR2 residues of the high-affinity T1-S18.45 TCR. The preferential selection of aromatic amino acids at position 51 (i.e., wild-type Tyr, Phe, and Trp) further supports the conservation at this position and may reflect a general requirement for bulky amino acids that can adapt to varying MHC structures or chemistry. However, position 31 showed a more diverse collection of allowable amino acids, from the chemical perspective, at this position (i.e. wild-type Gln, Cys, Ala, and Val). Nevertheless, the preferences for the wild-type residues in each case are consistent with their evolutionary pressures to retain these in the context of the Vα2:HLA-A2 interaction. This is not to
say, however, that other CDR loops or residues would not suffice to provide the very low energy of interaction required for MHC-restriction through positive selection\textsuperscript{13}. Clearly, the maintenance of the docking angle and general positions of each CDR loop in all wild-type TCRs and their high-affinity TCR counterparts suggests that the MHC interactions with CDR1 and CDR2 could collectively be capable of restraining the orientation of the complex, even in the absence of co-receptor (since the high-affinity TCRs described here were selected in the absence of CD8).

In a recent study comparing the crystal structures of G4 and E4 TCRs with their cognate ligand mutTPI-DR1, Deng et al. showed that CDR2\textalpha made identical contacts with the MHC helices in both structures but there was more plasticity or “wobble” in CDR1\textalpha loops, a finding that they attribute to the influence of the CDR3\textsuperscript{14}. Our study provides a corresponding binding energy analysis that is consistent with these structural findings. Furthermore, we suggest that the energetically-conserved CDR2\textalpha residue (Y51) provides sufficient binding energy to contribute significantly to the very low affinity interactions required for positive selection. Although our study does not examine the importance of TCR\textbeta chain residues or the impact of the co-receptor, crystal structures of the A6 TCR have suggested that the TCR\textalpha chain may dominate in the interaction with Tax/A2\textsuperscript{5,56-58}. The structures of the other two V\textalpha2-positive TCRs described here are not known; thus, it remains to be seen if their V\textbeta CDR1 and CDR2 play a more significant role than the A6 TCR.
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Figure 2.1  Yeast display and isolation of INR1-T1 (T1) single-chain TCR variable fragments (scTvs). (a) Schematic of scTvs fusions for the human TCR, T1, which recognizes MART-1 peptides in presented in the context of HLA-A2. (b) The T1 TCR was cloned as a scTv and expressed on the surface of yeast (left panels). Yeast surface levels of scTV fusions were monitored for expression of an N-terminal tag [hemagglutinin (HA), black line] with anti-HA antibody and goat anti-mouse IgG alexa 647 secondary antibody or secondary only as a control (gray). The negative peak is due to yeast that have lost plasmid, and serves as an internal control for each induced yeast sample. Cells were incubated with anti-Vβ16 and anti-Vα2 antibodies followed by goat anti-mouse IgG alexa 647 secondary antibody, or secondary only as a control (gray). Cells were incubated with MART-1 peptide (ELAGIGILTV)/HLA-A2 dimer at 100 nM. A random mutagenesis library was generated using the T1 scTv as a template and sorted with anti-Vβ16 antibody and goat anti-mouse IgG alexa 647 secondary antibody. Clone T1-S18 was isolated after two rounds of sorting (middle panels). Site-directed libraries were made in CDR3 loop regions of the stabilized T1-S18 clone. The high-affinity clone, T1-S18.45, was isolated following four rounds of sorting with MART-1 peptide (ELAGIGILTV)/HLA-A2 dimer (right panels, black line). Histograms are representative of three or more experiments.
Figure 2.2 | Yeast display of MART-1-reactive TCRs INRi-T1 (T1) and INRi (R1). (a) Sequence alignments of the scTvs generated from the INRi-T1 (T1) and INRi (R1) T cell clones. The Vα S49 residue that was engineered into the template for thermal stability is shown in magenta. The “FGXG” motif is indicated in the alignment in boxes in cyan. The C105 of R1 is indicated in yellow. TCR-variable residue numbering has been kept consistent with sequence alignments with previously published high-affinity scTvs. (b) Yeast displaying the R1 C105F mutant scTv was stained with a 1:10 and a 1:50 dilution of anti-Vβ14 antibody followed by APC-conjugated goat anti-mouse secondary antibody. No positive staining is detectable relative to the control (gray).
Figure 2.3 I Flow cytometry histograms of the selections of the T1 error-prone library with an antibody against TCR Vβ. The T1 error-prone library was sorted sequentially with anti-Vβ16 antibody for two FACS selections. Aliquots of yeast cells after each sort were incubated with a 1:50 dilution of antibody followed by APC-conjugated goat anti-mouse secondary antibody.
Figure 2.4 | Thermal denaturation of the T1-S18 scTv mutant. Yeast displaying the T1-S18 scTv were incubated at the indicated elevated temperatures prior to staining with a 1:50 dilution of anti-Vβ16 antibody followed by APC-conjugated goat anti-mouse secondary antibody.
Figure 2.5 | Sequence line-ups of T1 template scTv, stabilized T1-S18, and high-affinity T1-S18.45. Conserved immunoglobulin fold residues are in cyan. Green residues indicate stabilizing mutations and yellow indicate high-affinity mutations. The Va S49 residue that was engineered into the template for thermal stability is shown in magenta. TCR-variable residue numbering has been kept consistent with sequence alignments with previously published high-affinity scTvs. 
Figure 2.6 | Flow cytometry histograms of the selections of the T1-S18 CDR3 libraries. The combined T1-S18 CDR3 libraries were sorted sequentially with MART-1 (nonamer; AAGIGILTV)/HLA-A2 (a) or MART-1 (decamer; ELAGIGILTV)/HLA-A2 (b) for a total of 4 sorts. Aliquots of yeast cells after each sort were then incubated with 100 nM selecting MART-1 pepHLA-A2-Ig dimer followed by APC-conjugated goat anti-mouse secondary antibody.
Figure 2.7 I T1-S18.45 scTv stained with 9mer and 10mer MART-1 peptide variants. Aliquots of the yeast-displayed T1-S18.45 clone stained at the indicated concentrations with MART-1 (nonamer; AAGIGILTV)/HLA-A2-Ig dimer (a) or MART-1 (decamer; ELAGIGILTV)/HLA-A2-Ig dimer (b) followed by APC-conjugated goat anti-mouse secondary antibody.
Figure 2.8 | Purity and SPR of soluble MART-1-specific, single-chain TCR T1 and its engineered variants. T1 template, T1-S18, and T1-S18.45 were expressed in the E. coli pET28 expression system, refolded from inclusion bodies, and purified by Ni-column and size-exclusion chromatography. (a) SDS-PAGE of purified scTvs and molecular mass markers (M). (b) SPR trace of MART-1 (ELAGIGILTV)/HLA-A2 binding immobilized T1-S18.45. Fitted parameters ($K_D$, $k_{on}$, $k_{off}$) are shown in the inset. (c) Table showing the binding affinities of the T1-derived scTvs for variants of the MART-1 peptide.
Figure 2.9 | Binding of peptide-loaded antigen presenting cells by soluble T1-S18.45 scTv. (a) Titration of biotinylated T1-S18.45 scTv on antigen-presenting cell line T2 (HLA-A2+) pre-loaded with MART-1 peptide (1 μM) or null peptide, SL9 (1 μM). Cells were stained with 3.9 nM (tan), 7.8 nM (gray), 15.2 nM (red), 31.1 nM (purple), 62.5 nM (green), 250 nM (blue), or 1 μM (black) biotinylated T1-S18.45 scTv, followed by SA-PE. Data shown are representative of four experiments. (b) Mean fluorescence intensity (MFI) values of histograms in (a) are plotted versus scTv-biotin concentration.
Figure 2.10 | Crystal structures of Vα2-containing TCRs showing MHC contact positions in CDR1α and CDR2α loops. CDR1α residues D27, R28, and Q31 (a, c, and e), and CDR2α residues Y51 and S52 (b, d, and f) assessed in the alanine-scanning study are highlighted in the structures of the A6 (a and b), Mel5 (c and d), and DMF5 (e and f) TCRs, which all contain the Vα2 region. PDB files 1AO7, 3HG1, and 3QDG were used, respectively, in PyMol. TCR residue positions are indicated in black. MHC residues are italicized in gray. TCR-variable residue numbering has been kept consistent with sequence alignments with previously published high-affinity scTvs, not the numbering in PDB files19.
Figure 2.11 | Alanine-scanning mutagenesis of Vα2-containing single-chain TCRs. Binding titrations of yeast-displayed mutants of Vα2-containing single-chain TCR fragments (scTv): (a) A6-X15 specific for Tax/HLA-A2, (b) 868-Z11 specific for SL9/HLA-A2, and (c) T1-S18.45 specific for MART-1/HLA-A2. Alanine mutants were stained in triplicate with 8 nM, 40 nM, 200 nM, 1 μM, and 5 μM cognate pepHLA-A2 monomers followed by PE-conjugated streptavidin. Normalized percent max mean fluorescence intensity (MFI) is plotted against cognate pepHLA-A2 monomer concentration. Error bars represent standard deviations of triplicate experiments. (d) Fold changes in binding were determined by the scTv concentrations at one-half maximum wild-type binding from titrations in a–c. Error bars represent standard deviations of triplicate experiments.
Figure 2.12 | In vitro selected mutants of yeast display libraries of the MART-1-specific TCR, T1-S18.45. Yeast display libraries at position 31 in CDR1α (a) and position 51 in CDR2α (b) were generated, stained with 45 nM MART-1/HLA-A2, and selected by FACS. The top staining (8-10%) clones were isolated, and amino acid distribution was determined by 454 sequencing. Amino acid residues that were positively and negative selected are indicated on the y-axis as a function of the logarithm of amino acid frequency post-selection divided by the frequency pre-selection. A total of 11,736 and 13,600 clones from the unselected position 31 library and selected position 31 library were sequenced, respectively; a total of 6,806, and 24,532 from the unselected position 51 library and selected position 51 library were sequenced, respectively.
Figure 2.13 | Overlay of the structures of the A6:Tax/HLA-A2 complex and the high-affinity TCR A6-c134:Tax/HLA-A2 complexes. The wild-type A6:Tax/HLA-A2 complex crystal structure (PDB: 1AO7)\textsuperscript{5} is shown in gray with the CDR1\(\alpha\) and CDR2\(\alpha\) loops indicated in red. The CDR1\(\alpha\) and CDR2\(\alpha\) of the high-affinity mutant A6-c134:Tax/HLA-A2 (PDB: 4FTV)\textsuperscript{21} are overlayed with the wild-type loops in green. CDR1\(\alpha\) residues D27, R28, and Q31 and CDR2\(\alpha\) residues Y51 and Q31 that were assessed in our alanine-scanning study are indicated. TCR-variable residue numbering has been kept consistent with sequence alignments with previously published high-affinity scTvs, not the numbering in PDB files\textsuperscript{19}.
References


CHAPTER THREE

ENGINEERING HUMAN HIGH-AFFINITY T CELL RECEPTORS AGAINST WILMS’ TUMOR ANTIGEN BY YEAST DISPLAY

Abstract

Wilms’ Tumor Antigen (WT1) is a transcription factor that is overexpressed in many hematological and solid tumors. A peptide from WT1 and its variants are being developed for use in peptide- and dendritic cell-based cancer vaccines, and several ongoing clinical trials are using WT1-specific T cell receptors (TCRs) in adoptive T cell transfer for the treatment of leukemia. Higher-affinity TCRs may improve the efficacy of T cell targeting to the WT1 antigen as they could better recruit CD4\(^+\) helper T cells to the tumor. In this study, I describe the multi-step engineering of WT1-specific TCR variants for improved binding affinities for the WT1/HLA-A2 antigen by yeast display and directed evolution of single-chain variable fragments (scTvs or scTCRs). The high-affinity variant called WT1-D13.1.1 (\(K_D = 330\) nM) isolated following multiple rounds of mutagenesis and selection could be expressed at high levels in \(E.\ coli\) and used to detect WT1/HLA-A2 complexes on the surface of antigen presenting cells (APCs). The WT1-D13.1.1 TCR is also currently being examined in CD4\(^+\) and CD8\(^+\) adoptive T cell models.

Author’s contribution statement: The WT1-P20 and WT1-P22 TCR genes were obtained from the laboratory of Phil Greenberg at the University of Washington. Dan Harris (Kranz lab) performed follow-up studies utilizing the high-affinity WT1 TCR described here in adoptive cell therapy models. These studies are briefly mentioned in the final paragraphs of the discussion.
Introduction

Activation of the cell-mediated immune response begins with the recognition of a foreign antigen presented in the context of a self-MHC molecule on the surface of antigen presenting cells (APCs). Despite the ability of some cancer cells to evade the immune response, it has been demonstrated that in many cases the human immune system is capable of developing antigen-specific molecules, T cell receptors (TCRs) and antibodies, against tumors. Based on the isolation of T cells that recognize these tumors, numerous tumor-associated antigens (TAAs) have been identified and classified\(^1,2\). In 2009 the National Cancer Institute (NCI) ranked 75 of these antigens according to a list of weighted criteria (e.g. therapeutic function, immunogenicity, specificity, oncogenicity) in a pilot project for the “Acceleration of Translational Research\(^3\).” The top antigen listed in this project was Wilms’ Tumor Antigen (WT1).

In the 1990s, WT1 was initially classified as a tumor suppressor gene as it was the causative gene in autosomal-recessive pediatric kidney malignancy called Wilms’ tumor (WT)\(^4-8\). However, subsequent studies found that the WT1 transcription factor was often implicated in leukemia and in many solid tumors, and as a result, it was also classified as an oncogene\(^4,9\). In healthy individuals, WT1 expression is typically restricted to the urogenital system during embryogenesis\(^10\) and has been shown to be essential for kidney development in WT1 knockout mice\(^11\). Expression in adults is limited to discreet tissues at very low levels, and as a result it has been considered a promising target for immunotherapies\(^12\). In particular, its expression in leukemia cells surpasses its highest normal expression in adult tissues 10- to 1,000-fold\(^13,14\).
Additionally, WT1-specific CD8+ T cells have been shown to distinguish tumor cells overexpressing the antigen from normal cells and to specifically mediate killing of leukemia cells but not healthy CD34+ cells15. To my knowledge, there have not been any reports of autoimmunity in humans or mice using WT1-targeted immunotherapies12,16,17.

Several additional factors have suggested that WT1 is an ideal target for cancer vaccine and targeted cell-based immunotherapies. First, its overexpression is ubiquitous in hematological malignancies and solid tumors. To date, WT1 overexpression has been shown in over 36 types of tumors with a growing list of potential cancer types that could benefit from WT1-targeted therapeutics12. Second, loss of expression of WT1 in cancer cells leads to reduced proliferation or cell death, suggesting that the risk of tumor escape through the evolution of antigen-loss variants is relatively small18,19. Finally, WT1 appears to be highly immunogenic, inducing robust T-cell-20-25 and antibody-26-28 mediated adaptive immune responses. As a result, WT1 has been the focus of over 20 peptide- and dendritic cell-based clinical trials aiming to boost the adaptive immune response against a variety of hematological and solid tumors12.

WT1 vaccinations have focused largely on the HLA-A2-restricted nonomeric epitope spanning residues 126-135 of WT1 (WT1126-134: RMFPNAPYL) (Fig. 3.1) or modified variants that contain substitutions that are typically involved in improving HLA-A2 binding and thus MHC presentation29. Despite the high safety profiles in WT1-vaccine trials published to date, the clinical response rates have been modest, with overall objective response rates of 46% in solid tumors and 64% in hematological tumor
and detection of immunological responses in 35% and 68% of the patients evaluated, respectively. Hypotheses accounting for the lack of clinical benefit in a significant portion of patients treated included poor immunogenicity of vaccines, the compromised state of patients’ immune systems, immunosuppression by T_{regs} and inadequate T cell repertoires to generate WT1-specific TCRs. Because WT1 is a self-antigen it is likely that reactive T cell clones would be deleted during negative selection in the thymus, leading to minimal immunogenicity in response to WT1/MHC antigen presentation.

In order to address these limitations, strategies involving adoptive transfer of T cells expressing WT1-specific TCRs are currently under development. Accordingly, there is a need to isolate or engineer TCRs that direct specific recognition and activation of T cell to tumors that express WT1. Wild-type TCRs that have been isolated from human tumor infiltrating lymphocytes (TILs) to date have been of relatively low binding affinities (K_D = 1-300 µM). To compensate for low affinities, T cells have evolved co-receptors on their surfaces (CD4 and CD8) that synergistically bind to MHC molecules (class II and class I, respectively) along with TCRs in order to mediate potent activity in an antigen-specific manner. Notably, TCRs with high affinities (less than a K_D value of about 1µM) are able to mediate T cell responses in a co-receptor-independent manner such that recognition of class I MHC by TCR-modified CD4^+ T cells can mediate potent activation and tumor control due to greater functional avidity. It has been shown that TCRs of higher-affinity or avidity may have significant advantages in conferring persistence in vivo and the acquisition of memory T cell phenotypes: a requirement for potent anti-tumor responses.
In vitro directed evolution has been used to generate TCRs with higher affinity for a specific pepMHC, including yeast display\textsuperscript{36-38}, phage display\textsuperscript{39}, and T cell display\textsuperscript{40}. In all three approaches, the general process involves engineering wild-type TCRs that have low affinity for their cognate pepMHC for increased affinity by selection of libraries of site-directed mutants in the TCR loops that contact the pepMHC interface\textsuperscript{41}. These TCR loops, called complementarity determining region (CDR) loops, contact the pepMHC in a common diagonal docking orientation that has been observed in virtually all crystal structures of TCR:pepMHC structures to date, such that germline-encoded CDR2 regions are positioned primarily over the MHC helices and the highly diverse CDR3 regions encoded by the junction of somatically rearranged gene segments lie primarily over the peptide\textsuperscript{42,43}. CDR1 regions are also encoded by the germline V region, but are capable of forming specific contacts with peptide in addition to forming contacts with MHC helices\textsuperscript{44-48}.

Because of this conserved docking orientation, affinity maturation libraries have generally been targeted to CDR3 loops, as this might favor the highest level of peptide specificity possible\textsuperscript{38,49-53}. However, mutagenesis libraries in CDR1 and CDR2 loops have also yielded high-affinity TCRs that maintain peptide specificity\textsuperscript{39,54-56}. It has been proposed that this occurs through “CDR editing,” in which the residues in the diverse CDR3 loops modulate the interactions of V region TCR residues with pepMHC residues\textsuperscript{57}, or via “peptide editing,” in which TCR:pepMHC interactions are influenced by changes of the binding surface of the MHC depending on the peptide bound\textsuperscript{43}.
Over the last 15 years, the Kranz group has developed a strategy for engineering high-affinity $\alpha\beta$ TCRs in a format analogous to single-chain antibody variable fragments (scFv). Hence, the TCR variable regions that make up the antigen binding domains of each chain are expressed as a single-chain, connected by a flexible linker ($V_\alpha$-linker-$V_\beta$ or $V_\beta$-linker-$V_\alpha$); this form has been called single-chain TCR (scTCR) or single-chain TCR variable fragment (scTv). Recently, we showed that one of the human $V_\alpha$ regions, called $V_\alpha 2$ (IMGT: TRAV12 family), is exceptionally stable in the single-chain format and highly amendable to engineering by yeast display$^{58}$.

In the current study, I describe the engineering of $V_\alpha 2$-containing WT1-specific TCR variants by yeast display using multiple rounds of mutagenesis and selection. First, the template scTv was stabilized through introduction of random mutations selected for improved display on the surface of yeast$^{58,59}$. Using stabilized scTv templates, affinity maturation libraries were generated in CDR3 and CDR1$\alpha$ loops and these were selected for improved binding to WT1/HLA-A2. A scTv variant with modestly enhanced binding affinity was isolated from the CDR1$\alpha$ library, and this was used as a template for the generation of additional CDR3 libraries. From the CDR3 yeast display libraries, a high-affinity WT1-specific scTv was further isolated.

The highest affinity $V_\alpha 2$-containing WT1-specific scTv was used to confirm the amino acid preference and binding at CDR2$\alpha$ position Y51 using site-directed mutagenesis and a single-codon library approach, which I previously showed to be highly restricted in a panel of three other $V_\alpha 2$-containing scTv fragments (Chapter 2)$^{38}$. The high-affinity scTv could be expressed in *E. coli* and refolded from inclusion bodies.
to produce soluble scTv with a binding constant (K_D) of 330 nM as determined by surface plasmon resonance (SPR). The soluble, biotinylated scTv could also detect WT1/HLA-A2 complexes on the surface of APCs loaded with exogenous WT1 peptide. The high-affinity TCR has also been examined by our lab in various adoptive T cell approaches in mouse models.

**Materials & Methods**

*Antibodies, pepHLA-A2, and flow cytometry reagents*

Antibodies used to detect yeast surface expression included the following: anti-HA epitope tag (Clone HA.11; Covance), anti-c-myc epitope tag (A21281; Molecular Probes), anti-hVβ3 FITC antibody (Clone CH92; Beckman-Coulter), anti-hVβ3.1 FITC antibody (Clone 8F10; Thermo Scientific), anti-Vα2 monoclonal antibody generated in our laboratory (data not shown), goat-anti-mouse IgM APC (Life Technologies), goat-anti-mouse IgG F(ab’)2 AlexaFluor 647 secondary antibody (Invitrogen), goat anti-chicken IgG (H+L) AlexaFluor 647 secondary antibody (Molecular Probes), and Streptavidin-phycoerythrin (SA-PE, BD Pharningen).

Peptides that bind to HLA-A2 [WT1<sub>126-134</sub>: RMFPNAPYL, MART1<sub>26-35</sub> A27L: ELAGIGILTV, Tax<sub>11 – 19</sub>: LLFGYPVYV] were synthesized by standard F-moc (N-(9-fluorenyl)methoxycarbonyl) chemistry at the Macromolecular Core Facility at Penn State University College of Medicine (Hershey, PA, USA). For FACS and flow cytometry analysis, recombinant soluble dimeric HLA-A2-Ig fusion protein (BD™ DimerX) was used. Additionally, a monomeric HLA-A2-biotin reagent generated by the exchange of a
UV-cleavable peptide for another HLA-A2-restricted peptide in the presence of UV light was utilized for flow cytometry\textsuperscript{60,61}.

\textit{Cloning and expression of scTv in yeast display vectors}

TCR variable region fragments (scTv) were expressed in yeast display plasmid pCT302 (Vβ-L-Vα)\textsuperscript{62}, which contains a galactose-inducible AGA2 fusion allowing for growth in Trp media. Induction of the scTv gene involves growth of the transformed EBY100 yeast cells to stationary phase in selection media followed by transfer to galactose-containing media. The wild-type WT1-P20 and WT1-P22 scTv genes contained the highly stable Vα2 gene segment and a serine at position 49α that was previously shown to provide added stability in other scTvs\textsuperscript{58}. The template genes were synthesized by Genscript (Piscataway, NJ, USA) with codon optimization for yeast.

The WT1 specific TCR genes were isolated from CTL clones and obtained from our collaborator, Phil Greenberg, at the University of Washington. The genes were synthesized by Genscript, cloned as a single-chain format (Vβ-linker-Vα) and introduced into the yeast display vector for expression on the surface of yeast. The scTvs consisted of the variable contains attached by the linker region GSADDAKDAAKKDGKS\textsuperscript{58,63,64}. The scTv was introduced into the NheI and XhoI restrictions sites of pCT302.

\textit{Generation, display, and selection of mutated scTv yeast display libraries}

Error-prone PCR was used to generate random mutations libraries using WT1-P20 or WT1-P22 as a template, as previously described\textsuperscript{65}. The resultant libraries
contained about $9.6 \times 10^6$ and $2.3 \times 10^7$ independent clones, respectively, as
determined by plating limiting dilution aliquots of yeast after electroporation. CDR1\(\alpha\) and
CDR3 libraries were generated using splicing by overlap extension (SOE) PCR
spanning 4-5 adjacent codons at a time\(^6\). The resulting WT1-wt/D13/D15 CDR1\(\alpha\)
library yielded $3.1 \times 10^6$, and the resultant pooled WT1-D13.1 CDR3 libraries yielded $3.5$
$X 10^6$ independent clones, as judged by plating limiting dilution aliquots of yeast after
electroporation.

For the WT1-wt/D13/D15 CDR1\(\alpha\) library, pre-SOE PCR products were generated
utilizing the following primer pairs on three templates (WT-P22, WT1-D13, and WT1-
D15): 5' - GGC AGC CCC ATA AAC ACA CAG TAT -3' (Splice 4L) and 5' - ACG ATC
GCT ATA GGT GCA GTT CAA TGA TGC AAT AGC ACC TTC CGG GAC ACT TAA
TGG GCC GCT - 3', and 5' – ATT GCA TCA TTG AAC TGC ACC TGC ACC TAT AGC GAT CGT
NNS NNS NNS NNS TTC TTT TGG TAT AGA CAG TAC AGT GGC AAA TCC CCG –
3' and 5'- TAA TAC GAC TCA CTA TAG GG -3' (T7). SOE PCR was performed with
each corresponding pre-SOE along with both T7 and Splice 4L.

For the WT1-D13.1 CDR3 libraries (as well as the initial WT1-D13/D15 CDR3
libraries mentioned that did not yield improved variants), pre-SOE PCR products were
generated for each of the four libraries utilizing the following primer pairs: \(\beta1\): 5' - GGC
AGC CCC ATA AAC ACA CAG TAT -3' (Splice 4L) and 5' – TGC ACA CAG GTA CAT
GGA AGT TTG ATT GGT ACT AGC GCT TTC CAG AAT CAA ACT GAA ACG TTC
TTT – 3’, and 5’ – AGT ACC AAT CAA ACT TCC ATG TAC CTG TGT GCA NNS NNS
NNS NNS NNS GAA CAG TTT TTC GGC CCA GGT ACA AGA TTA ACG GTG – 3’ and
5'- TAA TAC GAC TCA CTA TAG GG -3' (T7); β2: Splice 4L and 5’ – TGC ACA CAG GTA CAT GGA AGT TTG ATT GGT ACT AGC GCT TTC CAG AAT CAA ACT GAA ACG TTC TTT – 3’, and 5’ – AGT ACC AAT CAA ACT TCC ATG TAC CTG TGT GCA AGC AGT TCC ATC NNS NNS NNS NNS GGC CCA GGT ACA AGA TTA ACG GTG – 3’ and T7; α1: Splice 4L and 5’ – GGC GCA CAG GTA AGT GGC GCT ATC TGA CGG TTG GCT ATC ACG GAT TAA CAG AGA GAC ATA CTG GGA – 3’, and 5’ – CAA CCG TCA GAT AGC GCC ACT TAC CTG TGC GCC NNS NNS NNS NNS NNS NNS NNS NNS NNS AAT ATG CTG ACC TTC GGT GGC GGT ACT CGC TTA ATG – 3’ and T7; α2: Splice 4L and 5’ – GGC GCA CAG GTA AGT GGC GCT ATC TGA CGG TTG GCT ATC ACG GAT TAA CAG AGA GAC ATA CTG GGA – 3’, and 5’ – CAA CCG TCA GAT AGC GCC ACT TAC CTG TGC GCC GCG AAT AAC GCG NNS NNS NNS NNS NNS TTC GGT GGC GGT ACT CGC TTA ATG – 3’ and T7.

Yeast libraries were made by homologous recombination in EBY100 yeast by electroporation of error-prone or SOE PCR products along with Nhel and Xhol digested pCT302. The libraries were induced in galactose-containing media (SG-CAA) for 48 hours, washed with 1 mL 1% PBS/BSA, and stained with antibodies or pepMHC reagents at the concentrations indicated in Tables 3.1, 3.2, and 3.3. Cells were washed (1 ml, 1% PBS/BSA), and the most fluorescent cells were selected using a FACS Aria (BD Bioscience) high-speed sorter.
**Isolation and staining of high-affinity clones**

Following selections, library clones were isolated by plating limiting dilutions. Colonies were expanded and induced in galactose-containing media (SG-CAA) for 48 hours, washed with 1 mL 1% PBS/BSA, and stained with various concentrations of peptide/HLA-A2 (pepHLA-A2) DimerX, goat-anti-mouse IgG F(ab’)2 AlexaFluor 647 secondary antibody, or various concentrations of UV-exchanged pepHLA-A2, SA-PE. Cells were washed (1 ml, 1% PBS/BSA) and analyzed on an Accuri C6 flow cytometer.

Plasmids were recovered using Zymoprep™ Yeast Plasmid Miniprep II (Zymo Research) and introduced back into *E. coli* via heat shock transformation into Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen). *E. coli* cells were expanded and plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen). Sanger sequencing was used to determine the sequences of individual scTv clones by sequencing relevant regions of the pCT302 plasmid.

**Site-directed mutagenesis of the WT1-D13.1.1 scTv to generate the WT1-D13.0.1 and WT1-D13.1.1 Y51A scTvs**

In order to revert the CDR1α loop of the WT1-D13.1.1 scTv to the wild-type (WT1-P22) sequence, three adjacent site-directed mutants were made in positions 29-31 of the CDR1α using a QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies): D29Vα, L30Sα, and G31Qα. Yeast cells displaying the resultant mutant, called WT1-D13.0.1, were titrated with WT1/HLA-A2-Ig dimers from 160 pM to 500 nM and analyzed by flow cytometry.
Additionally, the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) was used to mutagenize the WT1-D13.1.1 tyrosine at position 51\(\alpha\) to alanine. Yeast cells displaying the resultant mutant, called WT1-D13.1.1 Y51A\(\alpha\), were titrated with WT1/HLA-A2-Ig dimers at concentrations ranging from 0.1 to 100 nM and analyzed by flow cytometry.

**Yeast display library generation, selection, and 454 sequencing**

A degenerate (based on NNS codon composition) library was made at CDR2\(\alpha\) residue Y51\(\alpha\) of the WT1-D13.1.1 scTv using SOE PCR as described above. The library was stained with 100 nM WT1/HLA-A2-Ig dimer and sorted by FACS one time. The top staining (8-10\%) clones were isolated, plasmids were recovered and amino acid sequences were determined by 454 sequencing (Roche/454 GS FLX+ Sequencer) as previously described\(^3^8\).

**Expression in E. coli, refolding, and biotinylation of soluble scTv fragments**

The WT1-D13.1.1 scTv was introduced into the pET28a expression vector with a C-terminal AviTag (Avidity) using NcoI and EcoRI restriction sites (forward primer: 5’ - TAT ACC ATG GGC AGC AGC CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG CCG CGC GGC AGC GAT GTT AAA GTG ACA CAA TCT - 3’, and reverse primer: 5’ - T TTA GAA TTC TTA TTC GTG CCA TTC GAT TTT CTG AGC CTC GAA GAT GTC GTT CAG ACC GCC ACC ACC GTG GTG AGG CTT AAC CAT TAA - 3’. Plasmids were transformed into the BL21-DE3 expression cell line (NEB), expanded, and induced
for expression. Following induction, cells were passed through a microfluidizer (Microfluidics Corporation, Newton, MA, USA), inclusion bodies were isolated and protein was purified as previously described\(^6\). Soluble scTvs were refolded and purified with Ni-NTA agarose resin (Qiagen, Valencia, CA) followed by gel filtration (Superdex 200, GE Healthcare). Folded scTvs were biotinylated \textit{in vitro} (Avidity, BirA enzyme). Biotinylation was verified by a gel-shift assay with streptavidin by SDS-PAGE (data not shown).

\textit{Binding of WT1-D13.1.1 scTv measured by surface plasmon resonance}

The binding of purified refolded WT1-D13.1.1 to WT1/HLA-A2 was monitored by surface plasmon resonance (SPR) using a Biacore 3000 instrument. Kinetic and equilibrium binding data were determined by immobilizing biotinylated pepMHC monomers on a neutravidin-coated CM5 sensor chip to 400-800 response units. A null pepMHC molecule was immobilized to the reference cell. Purified soluble scTvs were diluted to various concentrations in Biacore buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20, pH 7.4) and flowed over the reference and experimental flow cells at 30 ml/min at 25°C. Binding of the scTv to the null complex was subtracted from the scTv binding to the experimental complex to correct for bulk shift and any non-specific binding. Additionally, data obtained from injections with no analyte were subtracted from each concentration. Curve fitting and determination of on-rates and off-rates were performed using BIAEvaluation 4.1.1 software. Equilibrium $K_D$ values were determined by calculating half-max values from non-linear regression analysis of plots.
of the maximum response units (RU) for each scTv concentration. Values from repeated experiments were averaged and reported with standard deviations (n = 3).

**Binding of WT1-D13.1.1 scTv to peptide-pulsed APCs**

HLA-A2+ human cell line, T2 was incubated at 37°C for 2-3 hours with 1 μM WT1 (RMFPNAPYL), MART-1 (ELAGIGILTV), or Tax (LLFGYPVVY) peptide. Cells were washed with 1% PBS/BSA, and incubated on ice for 1 hour with biotinylated scTv at various concentrations. Cells were washed again with 1% PBS/BSA, followed by incubation with SA-PE (1:100) for 30-45 min on ice. Cells were washed twice more and analyzed using an Accuri C6 Flow Cytometer.

**Results**

**Engineering a stabilized scTv against WT1/HLA-A2**

The WT1-P20 and WT1-P22 TCR clones were isolated and cloned from WT1-reactive T cell lines by Phil Greenberg and colleagues at the University of Washington. Both WT1-P20 and WT1-P22 used the highly stable Vα2 segment\(^{58}\) and the Vβ3 segment. In order to engineer WT1 TCRs for improved stability and affinity against the WT1/HLA-A2 cancer antigen using yeast display, the variable regions of these TCR genes were cloned as single-chain TCR fragments (scTvs or scTCRs) in the Vβ-linker-Vα orientation as a C-terminal fusion to AGA-2 yeast mating protein in the pCT302 yeast display plasmid (Fig 3.2). The linker used for both constructs was GSADDAKKDAAKKDGS\(^{58,63,64,68}\). Additionally, an N-terminal HA epitope tag and C-
terminal c-myc epitope tag were included in the construct flanking the TCR variable genes in order to probe for expression and possible truncations that could occur due to the introduction of premature stop codons during the selection process, respectively. It was previously shown by my group that the presence of a serine at position 49 in the Vα2 segment conferred additional stability to yeast-displayed TCR\(^{58}\); Both the WT1-P20 and WT1-P22 wild-type scTvs contained this amino acid in their wild-type sequences.

Yeast expressing the WT1-P20 (data not shown) and WT-P22 (Fig. 3.3; first column) were induced to express their respective scTv fragments. Both were positive when analyzed by flow cytometry following staining with anti-HA and anti-c-myc antibodies, indicating that the yeast expressed the full-length constructs. In order to probe for expression of individual TCR variable domains, monoclonal antibodies that recognize the Vα and Vβ domains were used. These included anti-hVβ3.1 FITC IgG (Thermo Scientific) and anti-hVβ3 FITC IgM (Beckman Coulter), and an anti-Vα2 monoclonal antibody generated in our lab. Temperature stability assays suggested that the two monoclonal antibodies against the Vβ3 domain recognized conformation epitopes (i.e. the denatured TCR was not bound). This justified the use of these antibodies as probes for folding and stability of the yeast displayed Vβ3 domain (data not shown). On the other hand, the Vα2 monoclonal antibody, which was generated in our lab, likely recognizes a linear epitope and thus would not provide a useful probe for properly folded Vα on the surface of yeast\(^{38}\).

Whereas both WT1-P20 (data not shown) and WT1-P22 (Fig 3.3) yeast clones showed staining with the anti-Vα2 antibody, staining was not observed with the anti-Vβ3
antibodies. This result suggested that the scTv construct was not folded properly on the yeast cell surface. Because scTv fragments lack TCR constant regions that stabilize the TCR on the surface of the T cell, scTv constructs typically require mutations to generate a stabilized form that can be expressed on the surface of yeast\textsuperscript{38,58,64,65,68,69}.

To generate a stabilized variant of the scTv fragments of WT1-P20 or WT1-P22 that would be amendable to engineering on the yeast cell surface, error-prone mutagenesis was used to create random libraries using each TCR as a template, and libraries were selected in tandem with the two Vβ3 antibodies (Table 3.1). Previous studies by our group with mouse TCRs 2C and 3L2 and human TCRs A6, 868, and T1 have shown that selection of scTv variants with enhanced expression and temperature stability on the surface of yeast correlate with increased soluble expression\textsuperscript{38,58,64,69}. The WT1-P20 and WT1-P22 error-prone mutagenesis libraries were subjected to three rounds of selection with the two anti-Vβ3 antibodies (conditions shown in Table 3.1).

Staining of the yeast populations following selections showed the emergence of positive populations (Fig 3.4). Because only a modest increase in positive staining was observed following three selections of the WT1-P20 library (Fig. 3.4a), only the WT1-P22 library, which showed a stronger positive population (Fig. 3.5b), was pursued further. After three selections of the WT1-P22 library with the anti-Vβ3 antibodies, individual yeast clones were isolated and assessed for improved stability as measured by increased surface levels with anti-Vβ3 antibodies (data not shown). Two clones, called WT1-D13 (Fig 3.3; second column) and WT1-D15 (not shown), showed improved binding with very similar staining profiles (Fig. 3.3b; second column). As a result, both
were used as templates for affinity maturation of the WT1-P22 scTv. The WT1-D13 and WT1-D15 scTv plasmids were isolated and sequenced (Fig. 3.5), and both contained four stabilizing mutations. Two of the mutations were common to both scTvs (D51Gβ and S32Pα), and the other two varied (S79Cα and N92Sα for WT1-D13; and I103Tβ and I50Vα for WT1-D15). Notably, these mutations are located in both CDR loops and framework regions. Stabilizing mutations in other engineered scTvs have occurred at the interface of the α and β V domains, at the exposed regions of the variable regions that are normally buried by the constant regions, or in CDR loops38,58,64,65,68,69.

**Engineering an affinity-enhanced scTv against WT1/HLA-A2**

Although affinity maturation libraries were initially constructed in the CDR3 regions of the stabilized WT1-D13 and WT1-D15 scTv clones, no clones with improved binding were isolated following three selections with WT1/HLA-A2-Ig dimers (data not shown). As a result a CDR1α affinity maturation library spanning four adjacent residues at positions 29-32 was made utilizing the wild-type (WT1-P22) and stabilized WT1-D13 and WT1-D15 clones as templates by splicing by overlap extension (SOE) PCR (Fig. 3.5)66.

The WT1-wt/D13/D15 CDR1α library was FACS sorted according to Table 3.2 for binding to WT1/HLA-A2-Ig dimers (BD DimerX). Following five rounds of selection by FACS with WT1/HLA-A2-Ig dimers, a weakly positive population began to emerge (Fig. 3.6a). Sequencing of ten individual clones following the fifth sort revealed that all had the identical sequence, which was called WT1-D13.1. WT1-D13.1 contained CDR1α
sequence at positions 29-32 that were DLGS, thus containing three mutations from the wild-type P22 sequence (i.e. V29Dα, S30Lα, and Q31Gα) and reverting the WT1-D13 stabilizing mutation S32Pα back to the wild-type serine (i.e. P32Sα) (Fig. 3.5). Additionally, an F48Sβ mutation was introduced near the CDR2β, likely as a result of a PCR-based error. The WT1-D13.1 clone showed only a modest improvement in binding to WT1/HLA-A2 (Fig. 3.3; third column).

Engineering a higher-affinity scTv against WT1/HLA-A2

In order to further improve the affinity of the WT1 scTv, CDR3 libraries were generated using the WT1-D13.1 clone isolated from the WT1-wt/D13/D15 CDR1α library as a template. The WT1-D13.1 CDR3 libraries were generated by splicing by overlap extension (SOE) PCR at five degenerate codons in each CDR: two libraries in the CDR3β loop [β1: positions 95-104 and β2: positions 103-107] and two in the CDR3α loop [α1: positions 91-100 and α2: positions 100-105]. Each WT1-D13.1 CDR3 library was introduced into the yeast display vector by combining the linearized pCT302 vector, WT1-D13.1 CDR3 PCR product (i.e. CDR3α1, CDR3α2, CDR3β1, or CDRβb2 library), and competent EBY100 yeast cells. The four resultant libraries were pooled, and the combined libraries were FACS selected for binding to WT1/HLA-A2-Ig dimers as described in Table 3.3.

After three rounds of selection by FACS with WT1/HLA-A2-Ig dimers, a positive population began to emerge (Fig. 3.6b). Following the third sort, ten individual clones were stained individually, and one, called WT1-D13.1.1 showed increased binding to
WT1/HLA-A2 (Fig. 3.3; fourth column). Sequencing revealed that WT1-D13.1.1 was derived from the CDR3β1 library and contained four CDR3β mutations that varied from the WT1-D13.1 template: S95Tβ, S97Nβ, I103Yβ, and N104Lβ (Fig 3.5).

*Binding analysis of WT1-D13.1 and WT1-D13.1.1 on the surface of yeast*

In order to assess the binding of the WT1-D13.1 and WT1-D13.1.1 clones isolated from selections of affinity maturation libraries, yeast populations displaying the WT1-D13.1 and WT1-D13.1.1 scTvs on their surfaces were titrated with two pepMHC reagents. Because the WT1-D13.1 scTv was lower affinity, it was titrated with WT1/HLA-A2-Ig dimers only (160 pM to 500 nM), in order to enhance signal through avidity effects (Fig. 3.7a). Yeast cells were then washed and analyzed by flow cytometry. A plot of the MFI versus WT1/HLA-A2-Ig dimer concentration suggested that the WT1-D13.1 scTv exhibited an EC50 value of greater than 1 µM (Fig. 3.7b). The higher affinity WT1-D13.1.1 scTv was titrated with both WT1/HLA-A2-Ig dimers and UV-exchanged WT1/HLA-A2 monomers expressed and purified from *E. coli*. The WT1/HLA-A2 dimers were assayed at 160 pM to 500 nM (Fig. 3.8a), and the monomers were assayed at 6.4 nM to 4 µM (Fig. 3.8b). Mean fluorescence intensity (MFI) were plotted for each histogram versus the concentration of the WT1/HLA-A2 complex. Values were normalized using nonlinear regression analysis and EC50 values of 25 nM and 240 nM were determined for dimer and monomer, respectively (Fig 3.8c,d). Typically, pepMHC dimers show half-maximum values at concentrations considerably lower than that of monomers due to avidity effects on the yeast cell surface.
Mutational analysis of the selected WT1-D13.1.1 CDR1\(\alpha\) loop

In order to determine if the four mutations derived from selection of the WT1-D13.1 CDR3 library were dependent on the WT1-D13.1 CDR1\(\alpha\) mutation framework, site-directed mutagenesis was used to revert the WT1-D13.1.1 CDR1\(\alpha\) to wild-type (i.e. positions 29-31 DLG to VSQ). The resultant scTv variant, called WT1-D13.0.1 was displayed on the surface of yeast and titrated with the WT1/HLA-A2-Ig dimer at concentrations ranging from 32 pM to 500 nM and compared to the WT1-D13.1.1 (Fig. 3.9). Where the WT1-D13.1.1 scTv bound even to concentrations as low as 32 pM, the WT1-D13.0.1 did not bind even at the highest concentration of WT1/HLA-A2-Ig dimer (500nM), indicating that the CDR1\(\alpha\) mutations were required to increase the binding affinity of the CDR3 mutations.

Evaluation of the role of CDR2\(\alpha\) residue Y51 of the WT1-D13.1.1 scTv

In Chapter 2, I described experiments with a panel of three V\(\alpha\)2-containing scTv fragments (A6-X15, 868-Z11, and T1-S18.45) that showed: (1) the germline codon residue Y51 had similar and significant contributions in binding to cognate pepMHC in all three scTvs (approximately 1.5-2 kcal/mol in free energy), and (2) the tyrosine was preferred at position 51 for cognate pepMHC binding using a single-codon library approach that mimics positive selection, although structurally similar amino acids (e.g. phenylalanine and tryptophan) were allowable\(^{38}\). In order to determine if this position was also important for WT1/HLA-A2-binding of WT1-D13.1.1, a site-directed alanine mutant was made at position 51 (i.e. Y51A\(\alpha\)) and titrated on the surface of yeast with
0.1-100 nM WT1/HLA-A2-Ig (Fig. 3.10a). Whereas the wild-type WT1-D13.1.1 staining with WT/HLA-A2-Ig dimer showed staining at concentrations as low as 160 pM (Fig. 3.8a), no significant binding was seen at the highest concentration tested of WT1/HLA-A2-Ig dimer (100 nM) in the WT1-D13.1.1 Y51A mutant (Fig. 3.10a), suggesting that position Y51 also played a critical role in binding in the WT1-D13.1.1 TCR.

A single-codon library (NNS) was generated in CDR2α position 51 and selected once with 100 nM WT1/HLA-A2-Ig dimer, as performed with the T1-S18.45 scTv in Chapter 2. Following collection of the top 8-10% binding clones by FACS, plasmids isolated from the enriched population of yeast were subjected to 454 high-throughput sequencing to determine the amino acid frequency selected at the degenerate position (Fig. 3.10b). As was seen with the T1-S18.45 scTv library, selections showed an enrichment of the wild-type tyrosine, as well as structurally similar amino acids, phenylalanine and tryptophan. Histidine was also preferred at position 51 in the WT1-D13.1.1 scTv library, perhaps due to the aromatic imidazole ring.

Expression and binding studies of the high-affinity WT1-D13.1.1 scTv

The WT1-D13.1.1 scTv gene was cloned into a pET expression vector and expressed in *E. coli*. scTv proteins were refolded from inclusion bodies and purified by Ni-affinity and size exclusion chromatography. Although capable of being expressed at high levels, the folded protein appeared less stable at room temperature compared to several other higher-affinity scTvs expressed by my group previously\(^{38,58}\). The purified scTv protein was used in surface plasmon resonance (SPR) experiments to determine
binding affinities. Soluble pepMHC monomers were immobilized to the surface of a neutravidin-coated CMF5 sensor chip (with a null pepMHC in the reference cell). The equilibrium binding constant ($K_D$) determined from the average of independent SPR experiments was $330 \pm 32$ nM, whereas the affinity determined from the kinetic constants was $240 \pm 290$ nM.

To show that the WT1-D13.1.1 scTv could recognize WT1/HLA-A2 on the surface of APCs, soluble WT1-D13.1.1 scTv was cloned with a C-terminal peptide substrate for BirA, and the purified protein was biotinylated *in vitro*\textsuperscript{58,70}. To assess binding, the human cell line T2 (HLA-A2\textsuperscript{+}) was incubated with 1 μM Tax, MART-1, or WT1 peptides and washed. Biotinylated WT1-D13.1.1 scTv was titrated on T2 cells pre-loaded without peptide (Fig. 3.11a), or with the null peptide Tax (4 nM to 1 μM) (Fig. 3.11b), null peptide MART-1 (4 nM to 1 μM) (Fig. 3.11c), or cognate peptide WT1 (4 nM to 1μM) (Fig. 3.11d). The cells were washed, incubated with SA-PE and analyzed by flow cytometry. Only cells loaded with WT1 peptide were bound by the soluble WT1-D13.1.1 TCR (Fig. 3.11d). Non-linear regression of the plot of MFI versus scTv concentration of the WT1 titration showed that the soluble scTv exhibited an EC$\textsubscript{50}$ value of 260 nM (Fig. 3.11e), consistent with SPR data.

**Discussion**

A study using T cells transduced with an affinity-enhanced TCR in an adoptive therapy model showed that T cells exhibited increased persistence and anti-tumor efficacy\textsuperscript{31}. Recently, our group\textsuperscript{71} and others\textsuperscript{72} have shown that CD4\textsuperscript{+} T cells expressing
engineered nanomolar affinity TCRs mediated more potent activity than their wild-type counterparts in the murine 2C model system. It has thus been proposed that high-affinity TCRs might be particularly effective in targeting the WT1/HLA-A2 cancer antigen, especially in light of recent clinical trials and mouse studies\textsuperscript{16,17,31}.

Recently Chapuis and coworkers in the Greenberg laboratory demonstrated the clinical efficiency of WT1-reactive CTLs in a limited adoptive T cell therapy trial in human patients with high-risk, post-bone marrow transplant leukemias\textsuperscript{16}. In this pilot study, 11 leukemia patients were given donor-derived WT1-reactive CTLs; in 4 of the 11 patients the WT1-reactive CTLs were pretreated with IL-21 to prolong persistence prior to infusion. In the 7 patients that receive CTLs without IL-21, leukemia was temporarily suppressed, although transferred CTLs did not persist past 14 days. In contrast, the 4 patients that received IL-21-pretreated CTLs survived without leukemia relapse for >30 months without additionally treatment or without exhibiting symptoms of graft-versus-host disease (GVHD). Additionally, the transferred T cells continued to persist and demonstrated phenotypes characteristic of a memory T cell population\textsuperscript{16}. Two phase I/II clinical trials using T cells transduced with WT1-specific TCRs against various types of leukemia are in progress at both the University College in London and the Fred Hutchinson Cancer Research Center (FHCRC) at the University of Washington (http://www.clinicaltrials.gov, NCT01621724 and NCT01640301, respectively).

In the current study, I engineered high-affinity variants of a WT1-reactive TCR, called WT1-P22, which retained specificity for WT1/HLA-A2. Unlike previous efforts in my lab in engineering both human and murine TCRs with enhanced affinities\textsuperscript{37,38,58,64},
the engineering of the WT1-D13.1.1 high-affinity scTv involved a multi-step engineering process in which different regions were mutagenized and the resultant variants were used as templates for further engineering. First, the WT1 TCRs were expressed as scTvs on the surface of yeast, and stabilized through the introduction and selection of random mutations. Although stabilization of TCRs as scTvs on the surface of yeast have typically shown the selection of mutations at the interface of variable regions or at the ablated interface between variable and constant regions \(^{38,58,64,65,68,69}\), several mutations in both the WT1-D13 and WT1-D15 clones showed mutations in CDR loops (Fig. 3.5). With the exception of the CDR2\(\beta\) D51G other stabilizing mutations in CDR loops were not selected or maintained in affinity matured variants, perhaps due to altering the binding specificity in the regions. Notably, during the first affinity maturation round (i.e. CDR1\(\alpha\) libraries) the resultant WT1-D13.1 clone had an additional PCR-based mutation just prior to CDR2\(\beta\) (i.e. F48S) that could have served to further stabilized the TCR. It was noted in purification of the high-affinity WT1-D13.1.1 scTv that \textit{E. coli} expressed and refolded scTv fragments were relatively unstable at room temperature compared to other scTvs I have generated in my laboratory (data not shown)\(^{38,58}\), perhaps due to the limited number of stabilizing mutations at regions where they are typically found in the high-affinity variant.

Binding affinity estimates were determined from yeast-displayed WT1-D13.1.1 (\(EC_{50} = 25\) nM and \(EC_{50} = 240\) nM for dimeric and monomeric pepMHC, respectively) and soluble preparations of the protein (equilibrium \(K_D = 330\) nM by SPR and \(EC_{50} = 260\) nM in T2 binding assays). Often, SPR values show higher affinities as there are no
wash steps as there are in flow cytometry assays of scTvs on the yeast cell surface\textsuperscript{38,58}, although the estimates for both forms of WT1-D13.1.1 were similar.

Following the isolation WT1-D13 and WT1-D15 clones, I generated affinity maturation libraries in CDR3 and CDR1\(\alpha\) regions using the wild-type and stabilized scTv as templates. Although no scTv variants were isolated from the combined CDR3 libraries, the CDR1\(\alpha\) library yielded a single variant, called WT1-D13.1, with improved binding to WT1/HLA-A2. Because WT1-D13.1 only showed modest improvement in binding to WT1/HLA-A2, further mutagenesis was performed in the same regions as in the initial WT1-D13/D15 CDR3 libraries. The resultant high-affinity scTv variant, WT1-D13.1.1, selected mutations in the CDR3\(\beta\) positions 95-104 of WT1-D13.1, whereas mutagenesis of this same region utilizing the WT1-D13/D15 libraries did not yield scTvs with improved binding.

To further explore if the CDR1\(\alpha\) mutations were required for the binding enhancement seen from affinity maturation of WT-D13.1 to WT1-D13.1.1, I reverted the selected CDR1\(\alpha\) loop back to the wild-type residues. This resulted in a complete loss in detectable binding suggesting that the CDR3 residues selected in the second round of affinity maturation (i.e. to generate WT1-D13.1.1 from WT1-D13.1) acted cooperatively with the mutated CDR1\(\alpha\) loop of WT1-D13.1. This could possibly account for why I did not isolate improved variants from the initial WT1-D13/D15 CDR3 libraries (i.e. perhaps no CDR3 mutants present in the library were capable of producing productive contacts with WT1/HLA-A2 in the context of the wild-type CDR1\(\alpha\) loop). These results suggest that multi-step selection schemes, focusing on one loop at a time, may prove a
productive strategy in redesigning the TCR:pepMHC interface by making a series of modest improvements in a step-wise fashion which in combination form a higher affinity TCR. However, in terms of mutagenizing TCR loop positions that come in close proximity to MHC helices, preventative measures should be taken to ensure that non-specific contacts through strong interaction with MHC in a peptide-nonspecific manner are not selected, perhaps through the use of robust negative selection strategies with panels of non-cognate pepMHCs that mimic the negative selection process of central tolerance. In this regard, it will be interesting to revert the CDR2β mutations (F48S and D51G) that were isolated as “stabilizing” variants in WT1-D13.1.1 in order to make sure that these do not increase the affinity for WT1/HLA-A2. As these were isolated for improved surface display with the anti-Vβ antibody, one would not expect them to increase affinity for pepMHC substantially.

Along the same lines, it has been proposed that affinity mutations in CDR1 and CDR2 loops might increase affinity by binding to MHC residues due to their relative positioning, and therefore mediate non-specific T cell responses due to stronger interactions with non-cognate pepMHC. In one of two recent adoptive cell therapy trials utilizing T cells engineered to target the MAGE-A3 cancer testis antigen with severe adverse effects, a CDR2-engineered TCR resulted in an off-target cardiac toxicity through recognition of an unrelated peptide derived from titin in the heart. It has been proposed that TCRs with affinity-enhancing mutations in CDR3 regions only would be less likely to cross-react since the germline-encoded TCR regions are similar to the parental TCR that has been subjected to selection in the thymus. I suggest
that restricting the CDR1α library design to the four positions that are nearest to the peptide in crystal structures of Vα2-containing TCRs\(^{44,46,47,77,78}\) may likewise provide a safety margin, as CDR1 regions have also been shown to make direct contacts to peptide residues in specific TCRs\(^{44-48}\) and selected residues could potentially mediate similar contacts. Although no structures are available for the WT1-D13.1 and WT1-D13.1.1 scTvs, the CDR1α is positioned over the N-terminus of the peptide in virtually all TCRs\(^{42,43}\). Accordingly, I predict that the acidic aspartate residue selected at position 29 of the CDR1α in WT1-D13.1 and WT1-D13.1.1 could potentially interact with the N-terminal arginine at position 1 of the WT1 peptide to mediate a direct, peptide-specific interaction (Fig. 3.1).

The use of some affinity- or avidity-enhanced TCRs could lead to unacceptable risk for use in adoptive T cell therapies due to a higher cross-reactivity potential due to affinity-enhancing mutations. However, additional factors play an important role in determining the safety of engineered TCRs. In particular the specificity of the antigen being targeted plays an important role. For example, in the other MAGE-A3 trial mentioned, off-target neurological toxicities were demonstrated due to T cell recognition of a highly similar epitope derived from the related antigen MAGE-A12 in the brain, which varied from the MAGE-A3 peptide only at the anchor residue at position 2 (i.e. V2M)\(^{76}\). In a separate trial with gene-modified CTLs specific for MART1/HLA-A2, on-target toxicities were demonstrated, presumably due to the expression of MART1 in normal melanocytes in the skin, eye, and ear\(^ {79}\). To date, targeting of the WT1 antigen in both cancer vaccine and cell-based immunotherapies has not demonstrated
autoimmune reactions\textsuperscript{12,16,17}, suggesting it is a desirable target for adoptive therapies that possibly include affinity-enhanced TCRs.

A recent study by Schmitt and colleagues\textsuperscript{17} proposed that the thymic selection process may be “overprotective,” and affinity-enhanced TCRs with affinities higher than the threshold of thymic selection (\( K_D \) values < 1 \( \mu \text{M} \)) may be safe for gene therapy in adoptive cell therapies. In this study, the efficacy and safety of affinity-engineered receptors were tested against two tumor antigens, WT1 and the differentiation antigen mesothelin, in a murine model. In the case of WT1, two WT1-specific TCRs were engineered for improved affinity using T cell display\textsuperscript{40}, and transduced into CD8\(^+\) T cells. As observed in previous studies with some high-affinity TCRs\textsuperscript{71,72}, TCRs with higher binding affinity showed enhanced activation as measured by cytokine release in \textit{ex vivo} assays. In order to demonstrate safety of the affinity-enhanced TCRs, CD8\(^+\) T cells were transduced with several WT1-specific TCRs, expanded, and infused into mice\textsuperscript{17}. CTLs transduced with the higher-affinity receptors were not apparently stimulated by murine self-antigens. Furthermore, normal tissues were not targeted by the engineered TCRs even following \textit{in vivo} activation of the WT1-specific CTLs through the introduction of \textit{Listeria} expressing the WT1 peptide. Notably, the H2-D\(^b\)-restricted WT1 peptide expressed in mice is identical to that in humans\textsuperscript{80,81}. Finally, to examine whether affinity-enhanced TCRs would be likely to be deleted during normal selection in thymus, hematopoietic stem cells were transduced with the affinity-enhanced TCR genes\textsuperscript{17}. Despite the absence of self-reactivity in the periphery, T cell progenitors originating from engineered stem cells with the same affinity-enhanced TCRs were negatively selected.
This suggested that the process of central tolerance has evolved to have a lower threshold for the deletion of self-reactive TCRs. Studies with affinity-enhanced TCRs against mesothelin yielded similar results, suggesting that affinity engineered TCRs, in the case of these two targets, may be safe for use despite basal levels of expression of the tumor peptides.

Despite these results, it is likely that TCRs of even higher affinity (i.e. $K_D$ values in the low nanomolar or picomolar range) would be more prone to off-target toxicities. Recent studies by our group and collaborators have utilized the well-characterized 2C mouse system to assess the role of TCR affinity for adoptive T cell therapies\textsuperscript{71,72}. In these studies, the wild-type 2C TCR ($K_D = 30 \ \mu\text{M}$ for foreign antigen SIY/K\textsuperscript{b}) and high-affinity variant m33 ($K_D = 30 \ \text{nM}$ for foreign antigen SIY/K\textsuperscript{b})\textsuperscript{36} were used to assess the anti-tumor response in a B16-SIY murine melanoma model. Notably, the m33 affinity is significantly higher than the engineered TCRs currently used in TCR gene therapy trials to date; e.g., the affinity of the DMF5 TCR ($K_D = 40 \ \mu\text{M}$) is $>1,000$-fold lower than m33\textsuperscript{47}. Where the CD8$^+$ T cells expressing the high-affinity m33 TCR were deleted \textit{in vivo}, CD4$^+$ cells expressing the m33 TCR were able to mediate a response against SIY-positive tumors and persist \textit{in vivo}\textsuperscript{72}. Additionally, in a follow-up study the m33 TCR showed a significant advantage over its wild-type 2C counterpart in mediating destruction of B16-SIY melanomas in CD4$^+$ T cells, resulting in long-term tumor control without tumor outgrowth\textsuperscript{71}. These data suggest that use of high-affinity TCRs, in CD4$^+$ T cells only, might provide additional efficacy and safety for gene therapy over the use of
CD8$^+$ T cells$^{71,72}$, particularly with TCR binding affinities ($K_D$) below 1 $\mu$M that allow for co-receptor independent T cell activation$^{33,34}$.

One strategy that has been proposed to take advantage of the properties of both CD4$^+$ and CD8$^+$ T cells for adoptive cell immunotherapies includes transducing separated populations of CD4$^+$ and CD8$^+$ T cells with high-affinity ($K_D \leq 1$ $\mu$M; such as WT1-D13.1.1) and slightly enhanced-affinity ($K_D > 1$ $\mu$M; such as WT1-D13.1) TCR variants, respectively, and using them together in adoptive cell infusions$^{34,71,72,82}$. Recently, we described a strategy using a library of 2C and m33 TCR variants that varied at a single CDR2$\beta$ position, Y46$\beta$, which when mutated to alanine shows a 100-fold decrease in binding in the m33 TCR$^{33}$. By allowing for any possible amino acid at the Y46$\beta$ position in either the wild-type 2C ($K_D = 30$ $\mu$M) and high-affinity m33 ($K_D = 30$ nM), a potential 10,000-fold range of TCRs that recognize SIY/K$^b$ could be assessed when introduced into CD4$^+$ or CD8$^+$ T cells and introduced into mice with SIY-positive tumors$^{82}$. Using high-throughput sequencing, it was shown that the highest affinity TCRs were preferentially expressed on the surface of CD4$^+$ cells, with the same TCRs being deleted in CD8$^+$ cells. Using a similar single-site mutagenesis strategy, it could be possible to generate antigen-specific TCR variants with lower affinities by mutating conserved residues in the CDR2 loops that could potentially decrease the TCR’s affinity for self-MHC helices$^{34,83}$.

In a recent study, I showed that an analogous residue in the human CDR2$\alpha$ (i.e. germline-derived, conserved residue Y51$\alpha$) in three human V$\alpha$2-containing scTvs, when mutated to alanine, exhibited a 10 to 15-fold or greater decrease in binding in all three
TCRs examined\textsuperscript{38}. Using a similar single-site library approach at CDR2\(\alpha\) position Y51\(\alpha\) in the human MART1-specific T1-S18.45 scTv in a yeast display library, I showed that selection for binding to MART1/HLA-A2 \textit{in vitro} resulted in range of potential single-site substitution spanning an affinity range of \(>1,000\) fold, as no binding was detected in the T1-S18.45 Y51A\(\alpha\) mutant when stained with concentrations up to 5 \(\mu\)M (wild-type \(K_D = \) 45 nM)\textsuperscript{38}. Similarly in the current study, mutagenesis of the WT1-D13.1.1 to generate a Y51A\(\alpha\) mutant led to a significant decrease in binding (i.e. no detectable binding at 100 nM WT1/HLA-A2-Ig binding). This suggests that a similar strategy could be used to generate WT1-D13.1.1 clones with decreased affinity for WT1/HLA-A2. Similarly, selection of single-codon WT1-D13.1.1 libraries at position Y51\(\alpha\) suggest many possible substitutions could potentially reduce affinity to a range optimal for directing activity in either CD4\(^+\) or CD8\(^+\) T cells\textsuperscript{34}.

Preliminary studies with the affinity enhanced WT1-D13.1.1 variable fragments in adoptive T cell models are ongoing. To date, a full-length TCR using the variable fragments of the WT1-D13.1.1 scTv with mouse constant regions, was expressed on the surface of murine primary T cells. As the WT1-epitope recognized in mice is identical to that recognized in humans\textsuperscript{80,81}, it provides the unique opportunity to address the safety features of the human high-affinity TCRs in both full length and chimeric antigen receptor (CAR) formats in a chimeric HLA-A2/D\(^b\) transgenic murine model.

In conclusion, the targeting of the WT1/HLA-A2 antigen using high-affinity TCRs, such as the WT1-D13.1.1 variant described in the current study or related mutants with modulated affinity properties could provide an efficacious strategy for directing adoptive
T cell therapies, particularly with CD4⁺ T cells. Additionally, because strategies to assess normal endogenous antigen expression in healthy tissues and associated cross-reactivity of affinity or avidity-enhanced TCRs is on the forefront of the development of adoptive T cell strategies⁸⁴, the WT1-specific engineered TCRs provide a convenient system to assess potential adverse effects of human TCRs in a murine model.

**Acknowledgements**

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Figure 3.1 | Crystal structure of WT1/HLA-A2. Top-down view of WT1/HLA-A2 upon which the TCR CDR loops dock. Although no crystal structures exist for the WT1-P20 or WT1-P22 clones that are used as templates for engineering in this study, virtually all TCRs contact pepMHC in an invariant diagonal docking orientation that positions the Vα region over the α2 MHC helix and the N-terminal end of the peptide, and the Vβ region over the α1 MHC helix and C-terminal end of the peptide. The WT1 peptide is pictured in blue, and the HLA-A2 helices are shown in gray from the WT/HLA-A2 crystal structure [PDB: 3HPJ]85. Amino acid sequences are indicated. The crystal structure is visualized with PyMOL software (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC)86.
Figure 3.2 | Sequence alignment of WT1-P20 and WT1-P22 template scTvs. The sequences of two WT1-specific TCRs isolated from WT1-reactive CTL lines by Greenberg and colleagues. The conserved immunoglobulin fold residues are shown in cyan, and the Va S49 residue that has been shown to confer additional stability is shown in magenta. Both TCRs were used as templates for error-prone yeast-displayed libraries, although only one, WT1-P22, yielded highly stabilized TCR clones on the surface of yeast. TCR-variable residue numbering has been kept consistent with sequence alignments with previously published human Vα2-containing scTvs.
**Figure 3.3 | Yeast display and isolation of WT1-P22 scTvs.**

(a) Schematic of the scTv fusion of the human TCR, WT1-P22, which recognizes WT1 peptide presented in the context of HLA-A2. (b) The WT1-P22 TCR was cloned as a scTv and expressed on the surface of yeast (first column). The scTv fusion was monitored on the surface of yeast for expression with an antibody against the HA epitope (first row). Antibodies that recognize the TCR variable regions were also used: anti-Vβ3 (Thermo Scientific) (conformation epitope; second row) and anti-Vα2 (linear epitope; third row). Binding to WT1/HLA-A2 was monitored through incubation of yeast with soluble WT1/HLA-A2-Ig dimers (fourth row). Finally, yeast were probed for possible truncations arising from premature stop codons by monitoring the C-terminal tag c-myc (fifth row). A random mutagenesis library was generated using the WT1-P22 scTv as a template and sorted with anti-Vβ3 antibody and goat anti-mouse IgG AlexaFluor 647 secondary antibody. Clones WT1-D13 (second column) and WT1-D15 (not shown) were isolated after three rounds of sorting. Site-directed libraries were made in the CDR1α loop region using the wild-type WT1-P22 and stabilized WT1-D13 and WT1-D15 clones as templates. The affinity-enhanced clone, WT1-D13.1, was isolated following five rounds of sorting with WT1/HLA-A2 dimer (third column). Finally, site-directed libraries were made in CDR3 loop regions of WT1-D13.1 for further affinity maturation. The high-affinity clone, WT1-D13.1.1, was isolated following three rounds of sorting with WT1/HLA-A2 dimer (third column). The negative peak is due to yeast that have lost plasmid, and serves as an internal control for each induced yeast sample. Gray indicates yeast cells stained with secondary antibody only. Histograms shown are representative of two or more experiments.
Figure 3.4 | Yeast display engineering of WT1-P22 scTv for stability. Flow cytometry histograms of the WT1-P20 (a) and WT1-P22 (b) scTv error-prone libraries after sorting with two antibodies that recognize conformation epitopes on Vβ3. The WT1-P20 and WT1-P22 error-prone libraries were sorted sequentially with Thermo hVβ3.1 FITC IgG and Beckman-Coulter (BC) hVβ3 FITC IgM antibodies, AlexaFluor 647 goat anti-mouse IgG and goat anti-mouse IgM APC, for a total of 3 sorts according to Table 3.1. Aliquots of yeast cells after each sort were then incubated with a Thermo hVβ3.1 FITC IgG, AlexaFluor 647 goat anti-mouse IgG. Gray indicates yeast cells stained with secondary antibody only. Histograms are representative of n=2.
Figure 3.5 | Sequences of engineered WT1-P22 scTvs. The sequences of the WT1-specific template (P22), stabilized (WT1-D13 and WT1-D15) and affinity-enhanced (WT1-D13.1 and WT1-D13.1.1) TCRs. The conserved immunoglobulin fold residues are shown in cyan, and the Va S49 residue that has been shown to confer additional stability is shown in magenta\textsuperscript{56}. Mutations isolated from error-prone libraries are underlined and shown in green; mutations that resulted from PCR-based mutations are underlined and shown in orange; mutations isolated from affinity maturation libraries are boxed and the library residues varied are shown in yellow. TCR-variable residue numbering has been kept consistent with sequence alignments with previously published human V\textalpha 2-containing scTvs\textsuperscript{56,58}.
Figure 3.6 | Affinity maturation of WT1-D13. (a) The wild-type WT1-P22 and stabilized clones WT1-D13 and WT1-D15 isolated from error-prone libraries were used as templates for an affinity maturation library in CDR1α. The WT1-wt/D13/D15 CDR1α library was sorted sequentially with WT1/HLA-A2-Ig dimer, APC-conjugated goat anti-mouse secondary antibody, for a total of five sorts according to Table 3.2. Aliquots of yeast cells after each sort were then incubated with 100 nM WT1/HLA-A2-Ig dimer followed by APC-conjugated goat anti-mouse secondary antibody. Gray indicates yeast cells stained with secondary antibody only. (b) The affinity-enhanced clone WT1-D13.1 isolated from WT1-wt/D13/D15 CDR1α library was used as a template for CDR3 libraries. The WT1 D13.1 combined CDR3 libraries were sorted sequentially with WT1/HLA-A2-Ig, APC-conjugated goat anti-mouse secondary antibody, for a total of three sorts according to Table 3.3. Aliquots of yeast cells after each sort were then incubated with 100 nM WT1/HLA-A2-Ig followed by APC-conjugated goat anti-mouse secondary antibody. Gray indicates yeast cells stained with secondary antibody only. Histograms are representative of n=2.
Figure 3.7 | Binding properties of the yeast-displayed WT1-D13.1 clone. Binding of the yeast-displayed WT1-D13.1 to WT1/HLA-A2-A2 dimers. (a) Flow cytometry histograms of the enhanced-affinity scTv WT1 D13.1 stained with various concentrations of WT1/HLA-A2-Ig dimer, followed by fluorescently labeled goat anti-mouse IgG AlexaFluor 647 secondary antibody. Histograms are representative of n=3. (b) Plot of the mean fluorescence intensity (MFI) values of histograms versus WT1/HLA-A2 dimer concentration. An approximate EC$_{50}$ value is indicated.
Figure 3.8 | Binding properties of the yeast-displayed WT1-D13.1.1 clone. Binding of the yeast-displayed WT1-D13.1.1 to soluble pepMHC reagents. (a) Flow cytometry histograms of the high-affinity scTv WT1 D13.1.1 stained with various concentrations of WT1/HLA-A2-Ig dimer, followed by goat anti-mouse IgG AlexaFluor 647 secondary antibody. Histograms are representative of n=3. (b) Flow cytometry histograms of the high-affinity scTv WT1 D13.1.1 stained with various concentrations of biotinylated WT1/HLA-A2 monomer, followed by SA-PE (1:100) secondary. Histograms are representative of n=2. (c) Plot of the mean fluorescence intensity (MFI) values of histograms versus WT1/HLA-A2 dimer concentration. An EC$_{50}$ value is indicated. (d) Plot of the MFI values of histograms versus WT1/HLA-A2 monomer concentration. An EC$_{50}$ value is indicated.
Figure 3.9 | Binding titrations of the WT1-D13.1.1 and WT1-D13.0.1 scTvs. The WT1-D13.1.1 scTv (a) and WT1-D13.0.1 scTv (b) titrated with WT1/HLA-A2-Ig dimers at concentration ranging from 32 pM to 500 nM, followed by goat anti-mouse IgG AlexaFluor 647 secondary antibody.
Figure 3.10 | Analysis of position Y51α of the WT1-D13.1.1 scTv. (a) A yeast-displayed site-directed Y51α mutant was generated using WT1-D13.1.1 scTv as a template. The resultant yeast population was titrated with 0.1-100 nM WT1/HLA-A2-Ig dimer and analyzed by flow cytometry on the surface of yeast. Concentrations of WT1/HLA-A2 are indicated in the legend. (b) In vitro selected mutants of yeast-display position 51α library of the WT1-specific TCR, WT1-D13.1.1. Yeast display libraries at position 51 of the CDR2α of WT1-D13.1.1 were generated, stained with 100 nM WT1/HLA-A2-Ig dimer, and selected by FACS. The top staining (8-10%) clones were isolated, and amino acid distribution was determined by 454 sequencing. Amino acid residues that were positively and negative selected are indicated on the y-axis as a function of the ratio of the logarithm of amino acid frequency post-selection divided by the frequency pre-selection. A total of 1,786 and 1,459 clones were sequenced pre- and post-selection, respectively. Bars with hash marks indicated that zero clones with the indicated amino acid were found post-selection, indicating they were strongly selected against.
Figure 3.11 | Soluble binding of the high-affinity WT1-D13.1.1 to WT1/HLA-A2 on the surface of APCs. The high-affinity TCR WT1 D13.1.1 was expressed in E. coli, refolded, and biotinylated in vitro. The soluble WT1-D13.1.1-biotin monomers were used to stain human T2 (HLA-A2*) cells following incubation with no peptide (a), null peptide Tax (b), null peptide MART-1 (c), or cognate peptide WT1 (d). (e) Plot of mean fluorescence intensity (MFI) versus concentration of titrated WT1-D13.1.1 scTv that show that the WT1-D13.1.1 scTv only bound to the WT1-loaded T2 cells and not to controls. Data is representative of n=2.
Table 3.1 | Sorting conditions for selection of stable scTv variants from WT1 error-prone libraries. The antibodies and dilutions used are indicated for selection of stable variants from the WT1-P20 and WT1-P22 error-prone libraries.

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<td>Thermo Scientific hVβ3.1 FITC (1:10) AlexaFluor 647 goat anti-mouse IgG (1:100) AND Beckman Coulter hVβ3 FITC IgM (1:10) Goat anti-mouse IgM APC (1:4)</td>
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<td>Thermo Scientific hVβ3.1 FITC (1:10) AlexaFluor 647 goat anti-mouse IgG (1:100) AND Beckman Coulter hVβ3 FITC IgM (1:10) Goat anti-mouse IgM APC (1:4)</td>
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<td>100 nM WT1/HLA-A2-Ig dimer (DimerX; BD Pharmingen) AlexaFluor 647 goat anti-mouse IgG</td>
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<td>100 nM WT1/HLA-A2-Ig dimer (DimerX; BD Pharmingen) AlexaFluor 647 goat anti-mouse IgG</td>
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<tr>
<td>4</td>
<td>100 nM WT1/HLA-A2-Ig dimer (DimerX; BD Pharmingen) AlexaFluor 647 goat anti-mouse IgG</td>
</tr>
<tr>
<td>5</td>
<td>200 nM WT1/HLA-A2-Ig dimer (DimerX; BD Pharmingen) AlexaFluor 647 goat anti-mouse IgG</td>
</tr>
</tbody>
</table>

Table 3.2 | Sorting conditions for selection of scTv variants with improved affinities for WT1/HLA-A2. The concentrations of soluble WT1/HLA-A2-Ig dimer and secondary antibody used are indicated for selections of the WT1-wt/D13/D15 CDR1α affinity maturation library.
### Table 3.3: Sorting conditions for selection of scTv variants with high-affinity binding to WT1/HLA-A2

<table>
<thead>
<tr>
<th>Sort</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 nM WT1/HLA-A2-Ig dimer (DimerX; BD Pharmingen) AlexaFluor 647 goat anti-mouse IgG</td>
</tr>
<tr>
<td>2</td>
<td>100 nM WT1/HLA-A2-Ig dimer (DimerX; BD Pharmingen) AlexaFluor 647 goat anti-mouse IgG</td>
</tr>
<tr>
<td>3</td>
<td>10 nM WT1/HLA-A2-Ig dimer (DimerX; BD Pharmingen) AlexaFluor 647 goat anti-mouse IgG</td>
</tr>
</tbody>
</table>

*Table 3.3 | Sorting conditions for selection of scTv variants with high-affinity binding to WT1/HLA-A2. The concentrations of soluble WT1/HLA-A2-Ig dimer and secondary antibody used are indicated for selections of the WT1-D13.1 CDR3 affinity maturation libraries.*
References


CHAPTER FOUR

ENGINEERING DESIGNER T CELL RECEPTORS THROUGH THE USE OF SINGLE-CHAIN T CELL RECEPTOR SCAFFOLDS

Abstract

Binding of a T cell receptor (TCR) to a peptide/major histocompatibility complex (pepMHC) is the key interaction involved in antigen specificity of T cells. The recognition involves up to six complementarity determining regions (CDR) of the TCR. Efforts to examine the structural basis of these interactions, and to exploit them in adoptive T cell therapies, has required the isolation of specific T cell clones and their clonotypic TCRs. Here we describe a strategy using in vitro, directed evolution of a single TCR to change its peptide specificity, thereby avoiding the need to isolate T cell clones. The human TCR A6, which recognizes the viral peptide Tax in complex with HLA-A2, was converted to TCR variants that recognized the cancer peptide MART1/HLA-A2. Mutational studies and molecular dynamics simulations identified CDR residues that were important in the specificity switch. Thus, in vitro engineering strategies alone can be used to discover TCRs with desired specificities.


Authors’ contribution statement: S.N.S. designed and performed experiments and Rosetta-based computational modeling under the guidance of D.M.K. J.L.B. and Y.W. designed and performed molecular dynamics simulations under the guidance of E.T. N.K.S. performed an independent SPR study under the guidance of B.M.B. Data interpretation, manuscript preparation, and manuscript editing were performed by all the authors.
Heterodimeric αβ T cell receptors are responsible for recognizing antigenic peptides presented in the context of a product of the major histocompatibility complex (MHC) on the surface of antigen presenting cells (APCs). The interaction of a TCR and a peptide/MHC (pepMHC) can drive the T cell into various states of activation, depending on the affinity (or dissociation rate) of binding. This recognition also operates during thymic development of a T cell in a process that selects for T cells with TCRs that bind with low affinity to self pepMHC complexes (positive selection), but deletes T cells with TCRs that bind too strongly to self pepMHC complexes (negative selection or tolerance)\(^1,2\). T cells exported to the periphery are thus positioned to discriminate between a normal, healthy cell and one that expresses aberrant pepMHC due to an infectious agent such as a virus or due to cell transformation to a cancerous state.

TCRs contain six complementarity determining regions (CDRs), three (CDR1, CDR2, and CDR3) in each α and β chain, that are involved in binding pepMHC ligands. Based on the solved TCR:pepMHC structures\(^3,4\), a common diagonal docking orientation positions the TCR such that the CDR1 and CDR2 loops encoded by the germline variable (V) region genes are typically positioned over the helices of the MHC. The CDR3 loops of each chain are encoded by nucleotides at the junctions of somatically rearranged gene segments, and these hypervariable regions are appropriately positioned over the peptide (reviewed in\(^3,4\)). Although it was originally thought that peptide specificity was determined in large part through interactions of CDR3 loops with the peptide, many studies have suggested that antigen specificity is
more complex. For example, in an early study of I-E\(^k\)-restricted TCRs that recognized distinct peptides, transplantation of CDR3 loops was not sufficient to confer peptide reactivity, even when the same V\(\alpha\) chains were used by the two TCRs\(^5\). Based on current thinking, specificity in the TCR:pepMHC interaction can occur through various mechanisms, including: 1) residues in the germline-encoded CDR1 loops that make direct contact with peptide\(^6\text{-}^9\), (2) CDR1 and CDR2 contacts with MHC that yield peptide-specific interactions indirectly by altering MHC contact with the peptide\(^4,10\), (3) CDR3 loops that modulate contacts between CDR1/CDR2 residues and MHC\(^11\text{-}^14\), and (4) peptide influences that impact MHC interactions with CDR1, CDR2, or even CDR3 residues\(^15\text{-}^17\).

Given the complexity of interloop interactions among CDRs, it is difficult to use computational approaches to predict the basis of peptide specificity by TCRs, let alone to design TCRs with novel specificities \textit{de novo}. Directed evolution has been used to engineer TCRs with large improvements in binding affinity, while maintaining antigen specificity\(^18\text{-}^23\). These studies have frequently targeted CDR3 regions for mutagenesis to accomplish affinity maturation, but mutations in CDR1 and CDR2 loops have also yielded improvements in affinity while maintaining peptide specificity\(^20\text{-}^22,24\). Furthermore, computational approaches have been used to guide improvements in TCR affinity, with only subtle effects on specificity\(^25\text{-}^28\).

Although affinity maturation of TCRs has been achieved, there have not been reports in which the specificity of a TCR has been changed to a completely different peptide using directed evolution (i.e., from the cognate peptide to a non-cognate
peptide). Here we describe the successful *in vitro* engineering of the human TCR A6 that recognizes a cognate nonameric peptide from the viral protein Tax, converting the TCR to one that specifically recognizes a non-cognate decameric peptide from the melanoma antigen MART1. A6 TCR residues in CDR loops that were predicted to be nearest the non-cognate peptides in structural models were mutated in yeast display libraries. A6 TCR libraries that contained degenerate residues in CDR1\(\alpha\), CDR3\(\alpha\), and/or CDR3\(\beta\) were selected with MART1/HLA-A2. One of the MART1-specific TCRs was further affinity matured to an affinity (\(K_D\)) of about 100 nM. While some of the MART1-selected TCRs lost detectable binding to the cognate antigen, Tax, another class of selected-TCRs were peptide non-specific. Modeling of the highly-restricted CDR residues present in these clones suggests that they acted by enhancing interactions with HLA-A2 helices.

To examine the underlying basis of the peptide specificity switch, we used site-directed mutagenesis and comparative molecular dynamics simulations. These findings reveal how multiple residues across different CDRs could act in concert to generate peptide specificity. More generally, the study shows that it is possible to use directed evolution and *in vitro* approaches to engineer TCRs with alternative specificities, opening the possibility for rapid discovery of TCRs against a large array of cancer, viral and autoimmune antigens.
Materials & Methods

Antibodies, pepHLA-A2, and flow cytometry reagents

Antibodies and streptavidin-conjugates used to detect yeast surface expression included: anti-HA epitope tag (Clone HA.11; Covance), anti-c-myc epitope tag (A21281; Molecular Probes), goat anti-mouse IgG F(ab’)_2 AlexaFlor 647 secondary antibody (Molecular Probes), goat anti-chicken IgG (H+L) Alexa Fluor 488 and 647 secondary antibodies (Molecular Probes), and streptavidin-phycoerythrin (SA-PE, BD Pharmingen). Peptides that bind to HLA-A2 [Tax_11–19: LLFGYPVYV, MART1_26–35 A27L: ELAGIGILTV, WT1_126–134: RMFPNAPYL, Survivin95–104: LTLGEFLKL, Survivin95–104 T2M: LMLGEFLKL, SL9/HIVgag77–85: SLYNTVATL, NYESO-Val157–165: SLLMWITNV, preproinsulin15–24: ALWGPDAAA, Tel1p549–557: MLWGYLQYV, MDM253–60:VLFYLGQY, HBV_Env183–191: FLLTRILTl, and gp100209–217: ITDQVPFSV] were synthesized by standard F-moc (N-(9-fluorenyl)methoxycarbonyl) chemistry at the Macromolecular Core Facility at Penn State University College of Medicine (Hershey, PA, USA). Additionally, a UV cleavable peptide, KILGFVFIJ, where J is the photolabile amino acid residue prepared by standard Fmoc-peptide solid phase synthesis using commercially available Fmoc-3-amino-3-(2-nitro)phenyl propionic acid as a building block, was synthesized at the University of Illinois Protein Sciences Facility as previously described^{29,30}.

HLA-A2 reagents include recombinant soluble dimeric HLA-A2-Ig fusion protein (BD DimerX), and expressed and refolded HLA-A2 monomers and tetramers. HLA-A2 heavy chain was expressed as inclusion bodies in E. coli and refolded in vitro with a
UV-cleavable HLA-A2 binding peptide and human β-2 microglobulin as previously described\textsuperscript{8,29-31}. The HLA-A2 heavy chain contained a biotinylation substrate sequence for \textit{in vitro} biotinylation (Avidity, BirA enzyme). Peptide exchange with HLA-A2-biotin monomers containing the UV-cleavable peptide was achieved by exposure to UV light in the presence of excess peptide\textsuperscript{29,30}. Monomers were converted to tetramers by incubation at a 4:1 molar ratio of HLA-A2 monomer:SA-PE. Staining of yeast cells ($10^6$) was performed on ice for 45-60 minutes, cells were washed with PBS/BSA (1%), and analyzed by flow cytometry with an Accuri C6 flow cytometer.

\textit{A6 RD1, RD1.5, and RD2 library design}

Candidate residues for degeneracy were determined by measuring which CDR loop positions would be most likely to allow for contacts with a variety of non-cognate peptides using Rosetta Backrub flexible backbone modeling algorithms (https://kortemmelab.ucsf.edu/backrub/\textsuperscript{32,33}). Using the wild-type (wt) and high-affinity (c134) A6:Tax peptide/HLA-A2 crystal structures (PDB: 1AO7 and 4FTV, respectively)\textsuperscript{8,34} as input, Rosetta was used to model HLA-A2 restricted MART1 peptides [MART1\textsubscript{26–35 A27L}: ELAGIGLTV (called “MART1” unless otherwise specified), MART1\textsubscript{27–35}: AAGIGILTV, and MART1\textsubscript{27–35 A28L}: ALGIGILTV] and peptides WT1: RMFPNAPYL, SL9/HIV-gag: SLYNTVATL, and Survivin: LTLGEFLKL using the Rosetta Multiple Mutation Mutagenesis Module\textsuperscript{33}. For modeling purposes, the residue at position “0” of the MART1\textsubscript{26–35 A27L} 10-mer peptide was omitted from the prediction. Mutated residues were given a 10Å radius of effect for the flexible backbone modeling. PyMOL
was used to visualize and examine overlays of the lowest energy conformation of each model (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC)\textsuperscript{35}, and CDR loop residues that were within hydrogen bonding distance (2.5-3.5 Å) of the MART1\textsubscript{26–35} A\textsubscript{27L} peptide residues were identified. Based on these models, the frequency of residues within this distance was used to select five residue positions for codon degeneracy (NNS) using the A6-X15 scTCR, that contains stabilizing mutations for the display on the surface of yeast, as a template\textsuperscript{36}. Residue numbering is consistent with crystal structures of A6:Tax/HLA-A2 (PDB: 1AO7)\textsuperscript{8} and A6-c134:Tax/HLA-A2 (PDB: 4FTV)\textsuperscript{34}. An intermediary library (RD1.5) was designed based on the same Rosetta models used to guide the generation of the RD1 library. Five degenerate positions (NNS) included the TCR\textalpha{} positions Q30, T98, D99 and S100, and TCR\textbeta{} position L98. In this library the four adjacent CDR3\textbeta{} loop residues in positions 99-102 of the A6-X15 scTCR were reverted to the wild-type A6 residues (AGGR) and were not varied. Additionally, the arginine at position 27 in CDR1\textalpha{} was mutated to alanine as Rosetta models suggested it could cause potential steric and/or electrostatic clashes if bulkier residues were selected residues at nearby degenerate positions. Alanine-scanning of the R27\textalpha{} of the A6-X15 scTCR only led to a 3-fold change in binding with Tax/HLA-A2 (Chapter 2)\textsuperscript{23}. A second generation library (RD2) was based on visual inspection of the A6:Tax (LLFGYPVYV)/HLA-A2 crystal structure, selecting residues that were in close proximity to MART1 (ELAGIGILTV)/HLA-A2 and WT1 (RMFPNAPYL)/HLA-A2 in the overlaid
crystal structures. In RD2, five codon positions (TCRα D26, G28, S100, and W101; TCRβ L98) were made degenerate (NNK) with significant variance from the RD1 library. Positions D26α and G28α were chosen primarily due to their proximity (<3.5 Å) to the R1 position of the WT1 peptide in the overlaid structures, and the S100α position was chosen due to its proximity (<1 Å) to the N5 position of the WT1 peptide in the overlaid structure. The W101α was varied as MD data had suggested that this position, if altered, could allow for more loop flexibility. Finally the L98β was varied as it participates in a key interaction driving the affinity and specificity of A6 for Tax. Based on results of RD1 library selections, TCRα residue 30 was generated as a binary position (wild-type glutamine or selected threonine) and positions 99-102 in CDR3β, were also binary (wild-type A6 residues AAGR or high-affinity A6-X15 MSAQ).

Generation, display, and selection of RD1, RD1.5, and RD2 yeast display libraries

The A6 libraries were expressed in the yeast display plasmid pCT302 (in orientation Vβ-L-Vα)37,38. The RD1 and RD1.5 libraries were synthesized by Genscript (Piscataway, NJ, USA), and the RD2 library by DNA2.0 (Menlo Park, CA, USA) using A6-X15 as a template, which included five framework mutations (S33A, E59D, N62D, N66K, K120I, all in the Vβ domain) and two CDR mutations (A52V and Q106L, both in the Vβ domain) that were isolated previously in a stability screen of the scTCR36. The RD1 library also contained four CDR3β mutations (A99M, G100S, G101A, and R102Q) that yielded higher affinity binding to Tax/HLA-A220. The RD1.5 library also had a TCRα R27A mutation that did not reduce binding substantially in a panel of Vα2-containing
high-affinity scTCRs (Chapter 2). All library constructs consisted of the variable TCR fragments attached by the linker region GSADAKKDAKKDGKS using the A6-X15 scTCR as a template. N-terminal HA and C-terminal c-myc epitope tags were added flanking the scTv gene to monitor for expression.

The RD1 library was synthesized by Genscript (Piscataway, NJ, USA) with an added C-terminal c-myc epitope tag, where regions indicated by “X” were made degenerate by NNS codons and “*” indicates a stop codon:

NAGVTQTPKFQVLKTGQSMTLQQACQDMNHEMYMAWYRQDPGMGLRLIHYSVGVGITD
QGDVPDGKYKVSRTTEDFPLLLSAAPSQTVFCASRPGXMSXQPELYFPGPRTLTV
TEDLINGSADAAKKDDAKDGKSQKEVEQNSGPLSVPEGAIASLNCTYSDRGXSFFW
YRQYSGBKPELISIYSNGDKEGRFTAQLNKASQYVSLLIRDSQPSDSATYLCAVTXX
SWGKLQFGAGTQVVTPDEQKLISEEQL**. The gene was codon optimized for both yeast and E. coli with 5' sequence TCT GCT AGC and 3' sequence CTC GAG ATC TGA.

For homologous recombination in yeast, pCT302 overhangs were added to the synthesized RD1 library using forward primer 5'-CAG GCT AGT GGT GGT GGT GGT TCT GGT GGT GGT TCT GGT GGT GGT TCT GGT GGT TCT GCT GAG AAT GCT GGT GTA ACA CAA ACG CCA A-3' and reverse primer 5'-GGA ACA AAG TCG ATT TTG TTA CAT CTA CAC TGT TGT TAA CAG ATC TCG AGT CAT TAT AAA TCT TCT TCA GAG ATC-3'. Yeast libraries were generated by homologous recombination in EBY100 yeast by electroporation of PCR products along with NheI and XhoI digested yeast display plasmid, pCT302. The resultant library size for RD1 was 6 X 10^6.
The RD1 library was induced in galactose-containing media (SG-CAA) for 48 hours, washed with 1 mL 1% PBS/BSA and stained with the following: Tax (LLFGYPVYV), MART1 (ELAGiGILTV), or WT1 (RMFPNAPYL) peptide/HLA-A2-Ig dimers, goat-anti-mouse IgG F(ab’)2 AlexaFluor 647 secondary antibody (1:100). Cells were washed (1 ml, 1% PBS/BSA), and the most fluorescent cells were selected using a FACS Aria (BD Bioscience). Selections were performed with Tax/HLA-A2-Ig dimer (sort 1: 20 nM, sorts 2-4: 10 nM), MART1/HLA-A2-Ig dimer (sorts 1-2: 500 nM, sorts 3-4: 100 nM, sort 5: 20 nM), and WT1/HLA-A2-Ig dimer (sorts 1-2: 500 nM, sorts 3-4: 100 nM). During the 3rd sort with MART1/HLA-A2 and WT1/HLA-A2, yeast cells were also stained with chicken anti-c-myc antibody, goat anti-chicken IgY AlexaFluor 488 secondary antibody and double positives were isolated in order to exclude truncated clones. Expression was monitored with an anti-HA epitope tag (1:50), goat-anti-mouse IgG F(ab’)2 AlexaFluor 647 secondary antibody (1:100), and anti-cmyc (1:50), goat-anti-chicken IgG (H+L) AlexaFluor 488 or 647 secondary antibody (1:100).

The RD1.5 Library was synthesized by Genscript (Piscataway, NJ, USA) with an added C-terminal c-myc epitope tag, where regions indicated by “X” were made degenerate by NNS codons and “*” indicates a stop codon:

NAGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMAWYRQDPGMGLRLIHYSVGVGIDTD
QGDVPDGYKVSRTTEDFPLLASSAPSQTSVYFCASRPGXAGGRPELYFGPCTRLTV
TEDLINGSADDAKDAAKKDQSGKEVEQNSGPlsVPEGAIASLNCTYSDAGSXSFFW
YRQYSGKSPSLEMSIYNSNGDKEDGRFTAQLNKASQYVSSLRDSQPSDSATYLCAVTXX
XWGKLQFGAGTQVVTPDEQKLISEEDL**.
The gene was codon optimized for both yeast and *E. coli*, and the following flanking DNA sequences were added which contained overlap with the T7 and Splice4L cloning primers. N-terminal DNA sequence: 5’ – GGC AGC CCC ATA AAC ACA CAG TAT GTT TTT AAG GAC AAT AGC TCG ACG ATT GAA GGT AGA TAC CCA TAC GAC GTT CCA GAC TAC GCT CTG CAG GCT AGT GGT GGT GGT GGT TCT GGT GGT GGT TCT GGT GGT GGT TCT GGT AGC – 3’, and C-terminal DNA sequence: 5’ – CTC GAG ATC TGT TAA CAA CAG TGT AGA TGT AAG GAC AAA GAC TTT GTA CCC ACT GTA TTA GGT CGT ACA AAA CAC AAT ATG ATC TTT AAT ATC ATT TCT CCG TAA ACA ACG TGT TTT CCC ATG TAA TAT CCT TTT CTA TTT TTC GTT CCG TTA CCA ACT TTA CAC ATA CTT TAT ATA GGT ATT CAG TTC TAT ACA CTA AAA AAC TAA GAC AAT TTT AAT TTT GCT GCC TGC CAT ATT TCA ATT TGT TAT AAA TTC CTA TAA TTT ATC CTA TTA GTA GCT AAA AAA AGA TGA ATG TGA ATC GAA TCC TAA GAG AAT TGA GCT CCA ATT CGC CCT ATA GTG AGT CGT ATT A. The delivered PCR product was amplified by PCR using the Splice4L and T7 primers, and yeast libraries were generated by homologous recombination in EBY100 yeast as described above42-45. The resultant library size for RD1.5 was 1.4 X 10^7.

The RD1.5 library was induced in galactose-containing media (SG-CAA) for 48 hours, washed with 1 mL 1% PBS/BSA, and stained with the following: Tax (LLFGYPVYV), MART1 (ELAGIGILTV), WT1 (RMFPNAPYL), Survivin (LTLGEFLKL), preproinsulin (PPI) (ALWGPDPAAA), and NYESO-Val (SLLMWITNV) peptide/HLA-A2-Ig dimers, goat-anti-mouse IgG F(ab’)2 AlexaFluor 647 secondary antibody (1:100). Cells were washed (1 ml, 1% PBS/BSA), and the most fluorescent cells were selected
using a FACS Aria (BD Bioscience). Selections were performed with Tax/HLA-A2-Ig dimer, MART1/HLA-A2-Ig dimer, and WT1/HLA-A2-Ig dimer (sorts 1-2: 200 nM, sorts 3-4: 100 nM); and Survivin/HLA-A2-Ig dimer, preproinsulin/HLA-A2-Ig dimer, and NYESO-Val/HLA-A2-Ig dimer (sorts 1-4: 200 nM). Expression was monitored with anti-HA epitope tag (1:50), goat-anti-mouse IgG F(ab')2 AlexaFluor 647 secondary antibody (1:100), and anti-cmyc (1:50), goat-anti-chicken IgG (H+L) AlexaFluor 488 or 647 secondary antibody (1:100). No clones with novel binding specificities were isolated during this screening progression.

The RD2 Library was synthesized by DNA2.0 (Menlo Park, CA, USA), where positions indicated by “X” were made degenerate by NNK codons, the positions labeled “1234” were binary allowing for A6 wild-type CDR3β loop AGGR or A6-X15 CDR3β loop MSAQ, the position indicated by “#” was binary allowing for either wild-type residue glutamine or mutated threonine, and positions indicated by “**” were stop codons:

NAGVTQTPKFQVLKGTQGSMTQQCAQDMNHAYMAYRQDPGMGLRLIHYSVGVGITD
QGDVPDGYKVSRTTEDFPLRLSAAASQTSVYFCASRGX1234PELYFGPGTRTLTVT
EDLINGSADAKKAHKDGKSTVEQNSGPLSVPEGAIASLNCTYSXRXS#SFFWYR
QYSGKSPELMISNLDGKEDGRFTAQLNKASQYVSLLIRDSQPSDSATYLCVTTDXX
GKLQFGAGTQVVTPDIEQKLISEDDL**.

The gene was codon optimized for yeast and the following flanking DNA sequences were added which contained overlap with the T7 and Splice4L cloning primers: N-terminal DNA sequence: 5’ – GCC AGC CCC ATA AAC ACA CAG TAT GTT
TTT AAG GAC AAT AGC TCG ACG ATT GAA GGT AGA TAC CCA TAC GAC GTT
CCA GAC TAC GCT CTG CAG GCT AGT GGT GGT GGT GGT TCT GGT GGT GGT GGT TCT GGT GGT GGT GGT TCT GCT AGC – 3’, and C-terminal DNA sequence: 5’ – CTC GAG ATC TGT TAA CAA CAG TGT AGA TGT AAC AAA ATC GAC TTT GTT CCC ACT GTA CTT TTA GCT CGT ACA AAA TAC AAT ATA CTT TTC ATT TCT CCG TTA ACA ACA TGT TTT CCC ATG TAA TAT CCT TTT CTA TTT TTC GTT CCG TTA CCA ACT TTA CAC ATA CTT TAT ATA GCT ATT CAC TTC TAT ACA CTA AAA AAC TAA GAC AAT TTT AAT TTT GCT GCC TGC CAT ATT TCA ATT TGT TAT AAA TTC CTA TAA TTT ATC CTA TTA GTA GCT AAA AAA AGA TGA ATG TGA ATC GAA TCC TAA GAG AAT TGA GCT CCA ATT CGC CCT ATA GTG AGT CGT ATT A. The delivered PCR product was amplified via PCR using the Splice4L and T7 primers, and yeast libraries were generated by homologous recombination in EBY100 yeast as described above. The resultant library size for RD2 was 2.4 X 10^8.

The RD2 library was induced in galactose-containing media (SG-CAA) for 48 hours, washed with 25 mL 1% PBS/BSA and stained with 5 µM Tax (LLFGYPVYV), MART1 (ELAGIGILTV), or WT1 (RMFPNAPYL)/HLA-A2 UV-exchanged HLA-A2 monomers. Magnetic bead selections were performed utilizing streptavidin MACS microbeads (Miltenyl Biotec), for a total of two selections using MACS LS columns on a QuadroMACS™ Separator (Miltenyl Biotec). Following two selections, the selected libraries were stained with the following: selecting peptide (Tax, MART1, or WT1)/HLA-A2-Ig dimer, goat-anti-mouse IgG F(ab’)2 AlexaFluor 647 secondary antibody (1:100). Cells were washed (1 ml, 1% PBS/BSA), and the most fluorescent cells were selected using a FACS Aria (BD Bioscience) high-speed sorter. Selections were performed with
1 nM and 100 nM peptide/HLA-A2 for selecting cognate antigen Tax, and selecting, non-cognate antigens MART1 (ELAGIGILTV) or WT1 (RMFPNAPYL)/HLA-A2, respectively. Expression was monitored with anti-HA epitope tag (1:50), goat-anti-mouse IgG F(ab')2 AlexaFluor 647 secondary antibody (1:100), and anti-cmyc (1:50), goat-anti-chicken IgG (H+L) AlexaFluor 647 secondary antibody (1:100).

Finally, in order to determine whether any additional clones with redirected specificities could be isolated from the three libraries described (RD1, RD1.5, and RD2), a combined selection was performed where the RD1, RD1.5 and RD2 libraries were combined in equal cell numbers adjusted for library diversity and selected with a panel of peptide/HLA-A2 antigens. In order to eliminate clones that bound to the cognate ligand, Tax/HLA-A2, the library population was stained with Tax/HLA-A2 monomers (5 μM) for 1 hour prior to adding anti-biotin MACS microbeads (Miltenyl Biotec) according to the manufacturer’s protocol and negatively selected with a MACS LS column on a QuadroMACS™ Separator (Miltenyl Biotec). Cells in the filtrate were expanded and an additional negative selection was performed with streptavidin MACS microbeads (Miltenyl Biotec) for a total of two negative selections. Negatively-selected cell populations were then subjected to positive selections with a panel of peptide/HLA-A2 antigens which included the following: WT1 (RMFPNAPYL), SL9/HIVgag (SLYNTVATL), Survivin-T2M (LMLGEFLKL), NYESO-Val (SLLMWITNV), preproinsulin (ALWGPDPAAA), MDM2 (VLFYLGQY), Tel1p (MLWGYLQYV), and HBV Env (FLLTRILTI)/HLA-A2 monomers for 2-3 additional selections with 2-5 μM peptide/HLA-
A2 monomers and alternating streptavidin and anti-biotin MACS microbeads (Miltenyl Biotec). No novel cross-reactive clones were isolated in this progression.

**Generation, display, and selection of RD-MART1 CDR3 yeast display libraries**

CDR3 libraries were generated by splicing by overlap extension (SOE) PCR spanning 5 adjacent codons at a time (two libraries in the CDR3β loop spanning residues 97-101 and 99-103; one library in the CDR3α loop spanning residues 98-102) using the RD1-MART1 scTCR clone selected from the RD1 library as a template. For the affinity maturation of the RD1-MART1 TCR, pre-SOE PCR products were generated for each of the four libraries utilizing the following primer pairs. β1: 5'- GGC AGC CCC ATA AAC ACA CAG TAT -3' (Splice 4L) and 5'- CGG ACG GGA AGC GCA GAA ATA CAC TGA GGT TTG AGA AGG TGC AGC GCT TAA CAG ACG CAG CGG -3', and 5'- ACC TCA GTG TAT TTC TGC GCT TCC CGT CCG NNK NNK NNK NNK CAG CCT GAA CTG TAC TTT GGT CCA GGC ACT AGA C -3' and 5'- TAA TAC GAC TCA CTA TAG GG -3' (T7); β2: Splice 4L and 5'- CGG ACG GGA AGC GCA GAA ATA CAC TGA GGT TTG AGA AGG TGC AGC GCT TAA CAG ACG CAG CGG -3', and 5'- ACC TCA GTG TAT TTC TGC GCT TCC CGT CCG NNK NNK NNK NNK CAG CCT GAA CTG TAC TTT GGT CCA GGC ACT AGA C -3' and 5'- TAA TAC GAC TCA CTA TAG GG -3' (T7); α: Splice 4L and 5'- CGT AAC CGC GCA CAA GTA TGT GGC CGA ATC GGA AGG CTG GGA GTC ACG AAT CAG CAA ACT AAC ATA CTG GC -3', and 5'- TCC GAT TCG GCC ACA TAC TTG TGC GCG GTT ACG NNK NNK NNK NNK NNK AAA CTG CAA TTT
GGT GCG GGC ACC CAG GTT GTG G -3' and T7. SOE PCR was performed with each corresponding pre-SOE along with both T7 and Splice 4L for each library.

Yeast libraries were generated by homologous recombination in EBY100 yeast as described above\textsuperscript{42-45}. Library diversity was confirmed at all 5 degenerate positions following sequencing of 6 clones from each library. The resultant library sizes were $\beta_1$: $2.1 \times 10^7$, $\beta_2$: $1.7 \times 10^7$, and $\alpha$: $1.1 \times 10^7$. Libraries were pooled in equal cell numbers in ratios reflecting relative diversity and expanded in SD-CAA media.

The combined library was induced in galactose-containing media (SG-CAA) for 48 hours, washed with 1 mL 1% PBS/BSA, and stained with MART1 (ELAGIGILTV)/HLA-A2-Ig dimer, goat-anti-mouse IgG F(ab')\textsubscript{2} AlexaFluor 647 secondary antibody (1:100). Cells were washed (1 ml, 1% PBS/BSA), and the most fluorescent cells were selected using a FACS Aria (BD Bioscience) high-speed sorter. Selection was performed with MART1/HLA-A2 (ELAGIGILTV)/HLA-A2-Ig dimer (sort 1: 200 nM, sort 2: 1 nM). Expression was monitored with anti-HA epitope tag (1:50), goat-anti-mouse IgG F(ab')\textsubscript{2} AlexaFluor 647 secondary antibody (1:100), and anti-cmyc (1:50), goat-anti-chicken IgG (H+L) AlexaFluor 647 secondary antibody (1:100).

\textit{Generation, display, and selection of RD2-WT1 yeast display libraries}

Following the third selection of the RD2 library with WT1/HLA-A2, two additional selections were performed prior to generating the RD2-WT1 yeast display libraries (described below). First, a negative FACS selection was performed by incubating the induced cell population with 200 nM Tax/HLA-A2-Ig dimer, goat-anti-mouse IgG F(ab')\textsubscript{2}
AlexaFluor 647 secondary antibody (1:100) and isolating cells that were negative for staining (i.e. sort 4). Next, a positive FACS selection with 200 nM WT1/HLA-A2-Ig dimer, goat-anti-mouse IgG F(ab’)2 AlexaFluor 647 secondary antibody (1:100) was performed (i.e. sort 5). Clones isolated from these two additional selection steps were all cross-reactive with the three pepMHC complexes tested (Tax/HLA-A2, MART1/HLA-A2, and WT1/HLA-A2) and contained scTCR sequences that resembled the cross-reactive clones examined after the third selection of the RD2 library (Fig. 4.2 and data not shown).

Several cross-reactive clones were used as templates to generate degenerate libraries based on NNK nucleic acid composition in the CDR3β positions 98-102. These included two clones isolated from the 3rd sort of the RD2 library (RD2-WT1-S3-1 and RD2-WT1-S3-5) and one clone isolated following the 5th sort of the RD2 library (RD2-WT1-S5-2). To generate libraries, pre-SOE PCR products were generated utilizing the following primer pairs. 5’- GCC AGC CCC ATA AAC ACA CAG TAT -3’ (Splice 4L) and 5’ – GCC AGG TCT TGA AGC GCA AAA GTA GAC AGA AGT TTG AGA TGG TGC TGC GGA CAA TAA TCT AAG AGG – 3’, and 5’- CT GTC TAC TTT TGG CTC AAG GAC TCA AGA CCT GGC NNK NNK NNK NNK NNK CCA GAA CTC TAC TTC GGG CCA GGA ACT AGA TTA ACC G – 3’ and 5’- TAA TAC GAC TCA CTA TAG GG -3’ (T7). SOE PCR was performed with each corresponding pre-SOE along with both T7 and Splice 4L for each library.

Yeast libraries were generated by homologous recombination in EBY100 yeast as described above42–45. Library diversity was confirmed at all 5 degenerate positions.
following sequencing of 10 clones, and the resultant library size was $7.2 \times 10^6$. The library was induced in galactose-containing media (SG-CAA) for 48 hours, washed with 25 mL 1% PBS/BSA, and stained with 5 µm Tax UV-exchanged HLA-A2 monomers.\textsuperscript{29,30} Negative selections were performed utilizing MACS microbeads (Miltenyl Biotec), for a total of two selections using streptavidin beads for the first selection and anti-biotin beads for the second using MACS LS columns on a QuadroMACS\textsuperscript{TM} Separator (Miltenyl Biotec). Following negative selections, two positive selections were performed by staining with 5 µm MART1/HLA-A2 or WT1/HLA-A2 UV-exchanged HLA-A2 monomers, followed by incubation with streptavidin (1\textsuperscript{st} selection) or anti-biotin (2\textsuperscript{nd} selection) MACS microbeads (Miltenyl Biotec) and passing through MACS LS columns on a QuadroMACS\textsuperscript{TM} Separator (Miltenyl Biotec). Following positive selections, one final negative selection was performed with 5 µM Tax/HLA-A2 monomers and streptavidin beads as before. Individual clones isolated following the final selection were stained individually, and plasmids were sequenced. As before, all isolated clones were cross-reactive with all three pepMHCs.

In a further attempt to reverse engineer RD2-WT1 isolates for WT1-specificity, RD2-WT1 isolated clones used for the CDR3\textbeta{} library (RD2-WT1-S3-1, RD2-WT1-S3-5, and RD2-WT1-S5-2) were used in combination with the non-truncated cross-reactive clones isolated from the selections of the RD2-WT1-CDR3\textbeta{} library with MART1 and WT1/HLA-A2 for templates for the RD2-WT1-CDR1\textalpha{} and RD2-WT1-CDR3\textalpha{} libraries. The RD2-WT1-CDR1\textalpha{} library was degenerate based on NNK nucleic acid composition at positions D27 and G29 (amino acid from wild-type sequence), and the RD2-WT1-
CDR3\(\alpha\) library was degenerate based on NNK nucleic acid composition at positions S99 and W100 (amino acid from wild-type sequence). To generate the libraries, pre-SOE PCR products were generated utilizing the following primer pairs. CDR1\(\alpha\): 5’- GGC AGC CCC ATA AAC ACA CAG TAT -3’ (Splice 4L) and 5’ – TGA GTA AGT ACA ATT CAA ACT AGC TAT CGC CCC TTC TGG AAC AGA TAG TGG ACC GGA GTT CTG – 3’, and 5’ – GCG ATA GCT AGT TTG AAT TGT ACT TAC TCA NNK AGA NNK TCT ACT TCC TTT TTC TGG TAC AGA CAA TAT TCT GG – 3’ and 5’- TAA TAC GAC TCA CTA TAG GG -3’ (T7); CDR3\(\alpha\): 5’- GGC AGC CCC ATA AAC ACA CAG TAT -3’ (Splice 4L) and 5’ – ATC TGT CGT CAC CGC ACA CAA ATA AGT AGC TGA ATC AGA AGG TTG ACT ATC TCT TAT TAG C – 3’, and 5’ – GCT ACT TAT TTG TGT GCG GTG ACG ACA GAT NNK NNK GGC AAA TTA CAA TTC GGT GCT GGG ACC CAG GTG G – 3’ and 5’- TAA TAC GAC TCA CTA TAG GG -3’ (T7). SOE PCR was performed with each corresponding pre-SOE along with both T7 and Splice 4L for each library.

Yeast libraries were generated by homologous recombination in EBY100 yeast as described above\(^{42-45}\). Library diversity was confirmed at the degenerate positions following sequencing of six clones. The resultant library sizes were 3.5 X 10\(^7\) for CDR1\(\alpha\) and 3.6 X 10\(^7\) for CDR3\(\alpha\). Libraries were pooled in equal cell numbers and expanded in SD-CAA media. The combined library was induced in galactose-containing media (SG-CAA) for 48 hours, washed with 25 mL 1% PBS/BSA and stained with 5 \(\mu\)M Tax UV-exchanged HLA-A2 monomers\(^{29,30}\). Negative selections were performed using MACS microbeads (Miltenyl Biotec), for a total of two selections using anti-biotin beads for the first selection and streptavidin beads for the second using MACS LS columns on a
QuadroMACS™ Separator (Miltenyl Biotec). Both the negatively selected library population and the unselected population were subjected to two positive selections by staining with 5 µM MART1/HLA-A2 or WT1/HLA-A2 UV-exchanged HLA-A2 monomers, followed by incubation with anti-biotin (1st selection) or streptavidin (2nd selection) MACS microbeads (Miltenyl Biotec) and passing through MACS LS columns on a QuadroMACS™ Separator (Miltenyl Biotec). Individual clones isolated following the final selections were stained individually, and plasmids were sequenced.

Isolation and staining of high-affinity clones

Following sorting with various selecting antigen, library colonies were isolated by plating limiting dilutions. Colonies were expanded and induced in galactose-containing media (SG-CAA) for 48 hours, washed with 1 mL 1% PBS/BSA and stained with various concentrations of peptide/HLA-A2-Ig dimer, goat-anti-mouse IgG F(ab')2 AlexaFluor 647 secondary antibody, or various concentrations of UV-exchanged peptide/HLA-A2 monomers29,30, SA-PE. Cells were washed (1 ml, 1% PBS/BSA) and analyzed on an Accuri C6 flow cytometer.

Plasmids were recovered using Zymoprep™ Yeast Plasmid Miniprep II Kit (Zymo Research) and introduced back into E. coli via heat shock transformation into Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen). E. coli cells were expanded and plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen). Sequences of individual clones were determined by Sanger sequencing.
Expression in E. coli, refolding, and biotinylation of soluble scTCR fragments

RD1-MART1 and RD1-MART1\textsuperscript{HIGH} were introduced into the pET28 expression vector with a C-terminal AviTag (Avidity) using Ncol and EcoRI restriction sites (forward primer: 5’ - TAT ACC ATG GGC AGC AGC CAT CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG CCG CGC GGC AGC AAT GCT GGT GTA ACA CAA ACG CC - 3’, Reverse primer: 5’ - T TTA GAA TTC TTA TTC GTG CCA TTC GAT TTT CTG AGC CTC GAA GAT GTC GTT CAG ACC GCC ACC GTC TGG AGT GAC CAC AAC CTG GGT - 3’. Plasmids were transformed into the BL21-DE3 cell line (NEB), expanded, and induced for expression. Following induction, cells were passed through a microfluidizer (Microfluidics Corporation, Newton, MA, USA), inclusion bodies were isolated, and protein was purified as previously described\textsuperscript{47}. Soluble scTCR were refolded and purified with Ni-NTA agarose resin (Qiagen, Valencia, CA) followed by gel filtration (Superdex 200, GE Healthcare). Folded scTCRs were biotinylated \textit{in vitro} (Avidity, BirA enzyme). Biotinylation was verified by gel-shift with streptavidin by SDS-PAGE (data not shown).

Binding of scTCR proteins measured by surface plasmon resonance

The binding of purified refolded scTCR proteins to cognate peptide/HLA-A2 was monitored with surface plasmon resonance (SPR) using a Biacore 3000 instrument. Kinetic and equilibrium binding data were determined by immobilizing biotinylated peptide/MHC monomers on a neutravidin-coated CM5 sensor chip to 400-800 response units. A null peptide/MHC molecule was immobilized to the reference cell as a control.
Purified soluble scTCRs were diluted to various concentrations in Biacore buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20, pH 7.4) and flowed over the reference and experimental flow cells at 30 ml/min at 25°C. Binding of the scTCR to the null complex was subtracted from the scTCR binding to the experimental complex to correct for bulk shift and any non-specific binding. Additionally, data obtained from injections with no analyte were subtracted from each concentration. Curve fitting and determination of on-rates, off-rates and kinetic-based $K_D$ measurements were performed using BIAEvaluation 4.1.1 software. Equilibrium $K_D$ values were determined by calculating half-max values from non-linear regression analysis of plots of the maximum response units (RU) for each scTCR concentration. Values from repeated experiments were averaged and reported with standard deviations. For equilibrium SPR values in this orientation, $n=3$ for RD1-MART1 and $n=2$ for RD1-MART1$^{\text{HIGH}}$. For kinetic SPR values in this orientation, $n=1$ for A6-X15 and RD1-MART1$^{\text{HIGH}}$.

Additionally an independent SPR experiment was performed in the reverse orientation (i.e. scTCR immobilized) to facilitate the use of multiple control pepMHC analytes. For this experiment, steady state binding was measured as previously described$^{48}$. Experiments were performed at 25°C with solution conditions of 10 mM HEPES, 3 mM EDTA, 150 mM NaCl, 0.005% surfactant P20, pH 7.4. RD-MART1 and RD-MART1$^{\text{HIGH}}$ were coupled directly to the surface of a CM5 sensor chip via amine linkage to a density of approximately 1000 RU. Soluble MART1/HLA-A2, Tax/HLA-A2, and gp100/HLA-A2 were injected over the surface in a series of concentration points until steady state was attained. Each pepMHC concentration series was injected twice.
Data was processed in BiaEvaluation 4.1 and globally analyzed in Origin 7.5. Values from repeated experiments in both orientations were averaged and reported with standard deviations.

*Binding of scTCR fragments to peptide-pulsed APCs*

HLA-A2+ human cell line, T2 was incubated at 37°C for 2-3 hours with 1 μM MART1 (ELAGIGILTV) or Tax (LLFGYPVYV) peptide. Cells were washed with 1% PBS/BSA, and incubated on ice for 1 hour with biotinylated scTCR at various concentrations. Cells were again washed with 1% PBS/BSA, followed by incubation with SA-PE (1:100) for 30-45 min on ice. Cells were washed twice more and analyzed using an Accuri C6 Flow Cytometer.

*Site-directed mutagenesis of scTCRs*

Site-directed mutants at position 98 of TCRβ (A6-X15 L98W and L98M, RD1-MART1 W98L, RD1-MART1$^{\text{HIGH}}$ W98L, and RD2-MART1-M98L) were made using a QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies). Yeast cells displaying the single-site mutants were titrated with cognate-peptide exchanged HLA-A2 SA-PE tetramers at 12.3 nM, 37.0 nM, 111 nM, 333 nM, and 1 μM and analyzed by flow cytometry. Values were normalized using non-linear regression analysis. Changes in binding affinity were approximated by determining the scTCR concentrations at one-half maximal wild-type binding. Independent experiments were performed with peptide/HLA-A2-Ig dimers with similar results (data not shown).
Rosetta sequence tolerance

Rosetta sequence tolerance algorithms\(^{49,50}\) were used to predict the specificity of certain residues in the TCR:pepMHC interactions. The crystal structures for A6-c134 (PDB: 4FTV) and DMF4 (PDB: 3QDG) were uploaded to the Rosetta server and used to generate an ensemble of 20 structures. Interacting partners taken into account of the sequence predictions included the TCR\(\beta\) chain (chain E), peptide (chain C), and HLA-A2 heavy chains (chain A), using self-interaction energies of 0.4, partner interacting energies of 1.0 and a Boltzmann Factor (kT) of 0.228, according to the published recommended values. The resultant frequencies are presented as a ranked list for the TCR\(\beta\) positions indicated.

Molecular dynamics simulations of A6 and modeled RD1-MART1

Four molecular complexes were modeled and simulated: A6:Tax/HLA-A2, A6:MART1/HLA-A2, RD1-MART1:Tax/HLA-A2, and RD1-MART1:MART1/HLA-A2. For A6:Tax/HLA-A2, the crystal structure of the high-affinity (c134) A6:Tax/HLA-A2 crystal structure (PDB: 4FTV)\(^{34}\) was used to construct the initial model for the simulation. The same crystal structure was also used as the template to model the RD1-MART1:MART1/HLA-A2 complex, since no experimentally derived structure is currently available for this complex. However, given the presence of MART1/HLA-A2 in other crystal structures showing variable orientations between the TCR and MHC components [TCRs DMF5 (PDB: 3QDG)\(^{6}\), DMF4 (PDB: 3QDM)\(^{6}\), and Mel5 (PDB: 3HG1)\(^{7}\)], four different orientations have been also modeled and simulated to investigate the
complexes that included MART1. These systems are referred to as the 4FTV/A6-c134, 3QDG/DMF5, 3QDM/DMF4, and 3HG1/Mel5 orientations, respectively.

In all the simulation systems that included Tax, the starting conformation was based on the crystal structure of A6-c134:Tax/HLA-A2\textsuperscript{34} (PDB: 4FTV; denoted as the 4FTV/A6-c134 orientation hereafter). The RD1-MART1 TCR structure was generated by introducing point mutations at respective sites in the A6-c134 TCR. The initial structures for Tax and MART1 peptides were taken from A6-c134:Tax/HLA-A2 (PDB: 4FTV)\textsuperscript{34} and DMF5:MART1/HLA-A2 (PDB: 3QDG)\textsuperscript{6} structures, respectively. An additional TCR-pepMHC complex for A6-c134 was constructed starting from the A6 wild-type crystal structure (PDB entry 1AO7), by \textit{in silico} mutation of positions 99-102 of the CDR3\(\beta\) loop (AGGR) to the A6-c134 sequence (MSAQ). In this system, the orientation of the TCR is similar to the 4FTV orientation, therefore this system is also considered to start from the 4FTV/A6-c134 orientation.

In order to reduce the size of the simulation system and thereby allow for better sampling and statistics of the dynamics and interactions at the interface, remote regions of MHC and TCR components were truncated. Thus, the MHC part was truncated to residues 1-182, and TCR \(\alpha\)- and \(\beta\)-chain were truncated to residues 1-119 and 30-122, respectively. To preserve the binding orientation and scaffolding of the structures, positional restraints were applied at the truncation sites; in MHC, C\(\alpha\) atoms of residues 30, 32, 96, 122, and 182 were harmonically restrained (force constant \(k = 1.0\) kcal/mol/Å\(^2\)) to their initial positions in all the simulations. Additionally, backbone carbonyl carbons of residue 119 in CDR\(\alpha\) and residue 122 in CDR\(\beta\) were harmonically...
restrained (force constant \( k = 1.0 \) kcal/mol/Å\(^2\)) in the following simulations: A6-c134:Tax/HLA-A2, A6-c134:MART1/HLA-A2 (4FTV/A6-c134 orientation), RD1-MART1:Tax/HLA-A2 and RD1-MART1:MART1/HLA-A2 (4FTV/A6-c134 orientation), in order to preserve the binding orientation of the TCR subunit.

All the simulations started from the 4FTV/A6-c134 orientation. Each simulation system was minimized for 1000 steps, followed by a 500-ps MD simulation under constant-volume, constant-temperature (NVT) conditions with all the backbone atoms restrained to their initial position (\( k = 10.0 \) kcal/mole/Å\(^2\)). This was followed by a 4-ns MD simulation under constant pressure, constant temperature (NPT) conditions, during which the restraints were gradually decreased and eventually eliminated completely.

After the initial equilibration phase, A6-c134:Tax/HLA-A2 and RD1-MART1:Tax/HLA-A2 were simulated each for another 100 ns of production runs, while, A6-wt MSAQ was simulated for 40 ns of production run. For MART1 simulations, in addition to the 4FTV/A6-c134 orientation described above, three additional initial models were constructed and simulated. Bound MART1/HLA-A2 is present in several crystal structures in complex with other MART1/HLA-A2-specific TCRs, in which the docking orientation of the TCR on the pepMHC varies. In order to examine all experimentally observed orientations of MART1/HLA-A2 binding to MART1/HLA-A2-specific TCRs, in addition to the orientation based on the A6-c134:Tax/HLA-A2 structure (4FTV/A6-c134 orientation), initial models representing orientations observed in PDB entries 3QDG\(^6\), 3QDM\(^6\), and 3HG1\(^7\), were also constructed using the protocol described below. We refer to these different orientations as 3QDG/DMF5, 3QDM/DMF4, and 3HG1/Mel5.
In order to generate the initial models for the three new orientations, in each case we started from the 4FTV/A6-c134 orientation described above. The model was then slowly morphed into the target orientation using biased simulations with system-specific collective variables (orientation quaternions). In the first step, a biasing potential with a force constant of \( k = 50,000 - 100,000 \text{ kcal/mol/radian}^2 \) was applied to the TCR subunit for 40 ns. This was then followed by a 60-ns relaxation simulation. In order to accelerate the relaxation to the new orientation, non-bonded interactions were scaled intermittently. The scaling factor was decreased from 1.0 to 0.8 and then increased back to 1.0, with a step size of 0.05. At each step, the structures were equilibrated for about 5 ns, followed by another 5 ns equilibration using a scaling factor 1.0. Once a relaxed structure was achieved, the systems were simulated for production runs: 100ns (for A6-c134:MART1/HLA-A2 in the 4FTV/A6-c134 and 3QDG/DMF5 orientations), and 170ns (for RD1-MART1:MART1/HLA-A2 in the 4FTV/A6-c134, 3QDG/DMF5, 3QDM/DMF4, and 3HG1/Mel5 orientations). The A6-c134:MART1/HLA-A2 complex in the 3HG1/Mel5 and 3QDM/DMF4 orientations was simulated for 50 ns only, since no significant binding interactions were evident for these complexes.

System preparation was done using VMD, and all the simulations were performed using NAMD. CHARMM27 force field was used for protein and ions. The simulations employed rigid bonds for bonds involving hydrogens (using SETTLE and RATTLE algorithms), with a 2 fs time step and periodic boundary conditions. For non-bonded interactions, a cut-off of 12 Å was used along with a switching function starting at 10 Å. Electrostatic interactions were computed using the particle mesh Ewald
method\textsuperscript{57}. Temperature was kept constant at 310 K using the Langevin method\textsuperscript{58}, with a damping coefficient of 1/ps. Pressure was maintained constant at 1.01325 bar with the isotropic Nosé-Hoover Langevin piston method\textsuperscript{59,60}, with a barostat oscillation period of 200 fs and damping time scale 50 fs.

\textit{MD SASA Analysis}

The solvent accessible surface area (SASA) was calculated for the heavy atoms in positions W101\textsubscript{α}, W98\textsubscript{β} (for RD1-MART) or L99\textsubscript{β} (for A6-134), using the backbone of Tax or MART1 and HLA-A2 as the environment. The results were normalized by the SASA of the same residue type in a vacuum. The first 1 ns of the simulations for A6 systems and the first 3 ns of the simulations of the RD1-MART1 systems were excluded from this analysis.

The interaction distances were calculated using the minimum distance between the terminal heavy atoms (e.g., nitrogens in arginine and oxygens in a glutamate) in each trajectory frame. Then the resulting data were plotted with bin widths ranging 0.1-0.2 Å. The peak location was used as the maximum likelihood interaction distance as shown in Fig. 4.22. The first 3 ns of the trajectories were excluded from the analysis.

\textit{MD simulation and analysis of DMF5 position F100\textsubscript{β}}

The simulation of DMF5:MART1/HLA-A2 complex was prepared using crystal structure with PDB identifier: 3QDG. TCR and MHC were truncated in order to focus on relevant regions: TCR\textsubscript{α} residues 1 - 108, TCR\textsubscript{β} residues 4 - 116 and MHC residues 1 -
182. In order to assess whether the insertion of the side-chain of F100β reoccurs, a rotation of 180° for χ1 dihedral angle and another rotation of 90° for χ2 dihedral angle were made for residue F100β. The following restraints were applied: The backbone of TCR and all heavy atoms in MART1/HLA-A2 were restrained with 1 kcal/mol/ Å² force constant. The resulting simulation system was first energetically minimized for 1000 steps and then run for ~4.3 ns under constant temperature, volume (NVT) condition.

For analysis, the root mean square deviation (RMSD) of the heavy atoms in the sidechain of F100β and the χ1 and χ2 dihedral angles were measured for comparison with the crystal structure. During the simulation, the aromatic ring of F100β flipped by 180°. For better comparison with the crystal structure, the CD1/CE1 and CD2/CE2 atom names were switched in the crystal structure data file during the data analysis.

**Backrub modeling of RD2-WT1**

Rosetta Backrub flexible backbone modeling algorithms (https://kortemmelab.ucsf.edu/backrub/)\textsuperscript{32,33} were used to model the RD2-WT1 mutations (D26I\textsubscript{α}, G28S\textsubscript{α}, S100R\textsubscript{α}, W101S\textsubscript{α}, and L98V\textsubscript{β}) into the wild-type A6 (PDB: 1AO7)\textsuperscript{8} crystal structure along with the wild-type Tax, MART1-10mer (ELAGIGILTV), and WT1 (RMFPNAPYL) peptides. Mutated residues were given a 10Å radius of effect for the flexible backbone modeling. For modeling purposes, the residue at position “0” of the MART1 10-mer peptide was omitted from the prediction. PyMOL software was used to visualize overlays of the lowest two energy conformation of each model (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC)\textsuperscript{35}, and the measurement
tool was used to determine which HLA-A2 residues were within 3Å of mutated RD2-WT1 residues.

**Results**

*Features of TCR A6 and the HLA-A2-restricted peptides Tax, MART1, and WT1*

In order to test whether the specificity of a TCR could be converted to a different MHC-restricted peptide by directed evolution, we used the human TCR A6, which was originally raised against the HTLV-1 peptide Tax (LLFGYPVYV)\(^{61}\). A6 was chosen due to its thorough structural and biochemical characterization\(^8,15,16,62,63\), and its prior expression as a stable single-chain TCR (Vβ-linker-Vα) in the yeast display system\(^36\).

Our goal was to convert the A6 TCR from binding the cognate peptide Tax to binding cancer-associated MART1 peptides (nonamer, AAGIGILTV and an anchor modified decamer, ELAGIGILTV) or WT1 (RMFPNAPYL)\(^{64-66}\). One of the advantages of the MART1 system is that MART1-specific TCRs have shown a preference for Vα2 (IMGT: TRAV 12-2)\(^{67}\), the same Vα region (i.e., CDR1α and CDR2α) used by A6. Additionally, the Vα2-containing MART1-specific TCR DMF5 targets MART1/HLA-A2 with a similar docking mode to the A6 TCR\(^7,28\). The MART1 peptides differ from Tax at every position except the primary anchor near the C-terminus (Fig. 4.1a,b), and the WT1 peptide differs fromTax at every position except positions 3 (F) and 8 (Y) (Fig. 4.1a,c). Notably, MART1 lacks the aromatic residues of Tax (i.e., F3, Y5, and Y8) and exhibits a distinct backbone configuration. The anchor modified MART1 decamer (ELAGIGILTV) binds with higher affinity to HLA-A2 than the nonamer (AAGIGILTV), although the two are
structurally divergent. MART1-specific TCRs often cross-react with both (Fig. 4.1b), and as a result the anchor-modified decamer was used for all selections due to its enhanced binding to HLA-A2. In summary, both MART1 and WT1 present unique surfaces to the TCR for examining the notion of whether a single TCR can be engineered to bind a non-cognate peptide.

In order to guide the mutagenesis strategy for the construction of A6 libraries, we examined, by modeling, which residues of the A6 CDR loops would be most likely to accommodate and provide binding energy to non-cognate peptides MART1 and WT1 in the HLA-A2 complex. Three MART1 peptide variants (MART1\textsubscript{26–35} \textsubscript{A27L}: ELAGIGILTV, MART1\textsubscript{27–35}: AAGIGILTV\textsubscript{27–35}, MART1\textsubscript{27–35}: ALGIGILTV\textsubscript{27–35} \textsubscript{A28L}), the HLA-A2-binding peptide WT1 (RMFPNAPYL), and several other peptides (e.g., SL9/HIV-gag (SLYNTVATL) and Survivin (LTLGEFLKL)) were modeled into the wild-type A6:Tax/HLA-A2 (PDB: 1AO7)\textsuperscript{8} structure using the Rosetta Multiple Mutation Mutagenesis Module\textsuperscript{32,33}. The lowest energy models for each peptide were examined with PyMOL\textsuperscript{35}, and candidate residues for generating libraries were chosen by determining the A6 TCR residues that were less than 3.5 Å from the peptide in the lowest energy conformations of various modeled TCR:pepMHC complexes. Because yeast display libraries can be generated to completely cover sequence diversity of up to five codons (NNS or NNK, 3\textsuperscript{25} = 3.3 x 10\textsuperscript{7}) or more, for the initial library (called RD1, rational design library 1) we selected five CDR positions that were the most commonly represented among the complexes within this distance: TCR\textsubscript{α} Q30, T98, and D99, and TCR\textsubscript{β} L98 and G101 (A101 in the A6-X15 template) (Fig. 4.1d).
Isolation of RD1 library variants that bound cognate peptide Tax or non-cognate peptide MART1

The RD1 library was generated by gene synthesis (Genscript), using as a template the single-chain (Vβ-linker-Vα) TCR called A6-X15, that contained four CDR3β mutations that conferred high-affinity for Tax/HLA-A2 and one CDR3β mutation that conferred increased stability for yeast display in the CDR3β (Fig. 4.2)36. In order to determine whether the RD1 library contained mutants that bound to MART1 or WT1, as well as to verify that the library contained mutants that bound to Tax, FACS was used for selections with Tax/HLA-A2-Ig, MART1/HLA-A2-Ig (using the anchor-modified decamer peptide), and WT1/HLA-A2-Ig dimers. As expected, the unselected RD1 library did not show detectable positive peaks with any ligand, but a positive population began to emerge for Tax/HLA-A2 and MART1/HLA-A2 after the second and fourth sorts, respectively (Fig. 4.3a,b). A positive peak did not emerge with WT1/HLA-A2 even after the fifth sort (data not shown) and thus only the Tax and MART1-reactive clones were pursued further.

Six clones isolated from the RD1 library following the fourth sort with Tax revealed that 2 of 6 had identical amino acid sequences to A6-X15 (although the nucleotide sequences/codons varied) and 4 of 6 had a threonine substitution at position 30 in CDR1α (Fig. 4.2 and Fig. 4.4). The similarities to A6-X15 suggest that there was strong selection for these residues in conferring high-affinity Tax binding. In addition, emergence of highly restricted residues through successive sorts also argued that the final high-affinity clones were evolved at these positions to optimize binding (Fig. 4.4).
To determine if the TCRs selected for binding to the Tax peptides exhibited specificity in their reaction with the selecting peptide, RD1-Tax-1 (Q30\(\alpha\), T98\(\alpha\), D99\(\alpha\), L98\(\beta\), and A101\(\beta\)) was titrated with various concentrations of Tax and MART1/HLA-A2-Ig dimers at concentrations ranging from 4 to 500 nM (Fig. 4.5a, top panels). The Tax-selected TCR, RD1-Tax-1, bound only to the Tax complex, and not to the MART1 complex, with half-maximal binding of Tax/HLA-A2-Ig dimer at approximately 110 nM (Fig. 4.5b). Five clones isolated from MART1 selection of the RD1 library following the fifth sort were also sequenced. All five clones had identical nucleotide sequences that encoded the following mutations: T30\(\alpha\), K98\(\alpha\), Y99\(\alpha\), W98\(\beta\), and G101\(\beta\) (called RD1-MART1) (Fig. 4.2 and Fig. 4.6). Except for T30\(\alpha\) and G101\(\beta\), these mutations differed with both the A6-wt and A6-c134 TCRs. Sequencing of clones following successive sorts showed the emergence of selected residues (K/R) at position 98 of CDR3\(\alpha\) during the third sort (Fig. 4.6). To determine if the TCR selected for binding to MART1 exhibited specificity, the RD1-MART1 clone was titrated with various concentrations of the MART1 and Tax/HLA-A2-Ig dimers (Fig. 4.5a, bottom panels). RD1-MART1 bound only to the MART1 complex, and not to the Tax complex, with half-maximal binding at approximately 130 nM (Fig. 4.5b). Thus, a higher affinity TCR mutant against a distinctly different peptide could be isolated from a degenerate library of a single TCR.

**Affinity maturation of the MART1-selected RD1 scaffold variant**

In order to determine if RD1-MART1 could be engineered for even higher affinity and yet retain its specificity, degenerate libraries (NNK) were made in the CDR3 loops
of RD1-MART1 spanning 5-codon regions in CDR3α residues 98-102 and CDR3β residues 97-101 and 99-103 (Fig. 4.7). Two rounds of selection were performed with the pooled libraries, first with 200 nM MART1/HLA-A2-Ig dimers and then with 1 nM MART1/HLA-A2-Ig dimers, a concentration below the detectable limit for staining the parental RD1-MART1 (Fig. 4.5b). Each sort contained a positive population of yeast (Fig. 4.3c), and ten clones from the second sort were sequenced. Eight clones contained the parental RD1-MART1 sequence, one clone contained a Q81K PCR-based mutation in the Vα region, and one clone, called RD1-MART1HIGH, had three mutations in CDR3β that differed from the parental sequence (S100A, Q102G, and P103V) (Fig. 4.2 and Fig. 4.7).

RD1-MART1HIGH retained specificity for MART1 and showed significant binding with monomeric MART1/HLA-A2, whereas the RD1-MART1 only showed binding at the highest monomer concentrations (Fig. 4.5c and data not shown). Staining of RD1-MART1HIGH with non-selecting cognate peptide Tax/HLA-A2 and other non-cognate peptides WT1 (RMFPNAPYL)/HLA-A2 and Survivin (LTLGEFLKL)/HLA-A2 showed no detectable signal even with 500 nM peptide/HLA-A2-Ig dimers, over 500-times the concentration of MART1/HLA-A2-Ig dimers that yielded detectable staining (Fig. 4.8).

**Design and selection of a second A6 scaffold library, RD2, with Tax, MART1, and WT1**

To determine if alternative diversity in the A6 scaffold could be used to generate TCR mutants that are specific for MART1/HLA-A2 or WT1/HLA-A2, a second library, RD2, was designed with five degenerate (NNK) positions: TCRα D26, G28, S100, and
W101 and TCRβ L98, a binary position at TCRα 30 (Q30 or T30) and a binary region in TCRβ 99-102 (AGGR or MSAQ) (Fig. 4.2 and Fig 4.9a). RD2 was screened with three pepMHC ligands, Tax/HLA-A2 (LLFGYPVYV; cognate), MART1/HLA-A2 (ELAGIGILTV; non-cognate) and WT/HLA-A2 (RMFPNAPYL; non-cognate), using two MACS selections followed by one round of FACS. Selections with the cognate antigen, Tax, and both non-cognate antigens, MART1 and WT1, showed the emergence of positively staining populations (Fig. 4.10).

Following selection with Tax/HLA-A2, six colonies were sequenced and analyzed. Although all six clones bound to Tax/HLA-A2 but not to MART1/HLA-A2 (data not shown), the two clones RD2-Tax-1 and RD2-Tax-2 showed improved staining in comparison to the high-affinity A6-X15 TCR36 when stained with Tax/HLA-A2-Ig dimers and monomers (Fig. 4.9b, left panels and data not shown). All six clones differed in sequence (Fig. 4.2), but each retained the four CDR3β residues MSAQ at TCRβ positions 99-102 (rather than the wild-type binary option AGGR) that were present in the high-affinity A6-X15 TCR. Two residues that were among the positions of complete degeneracy in the library, CDR3α W101 and CDR3β L98, were also highly restricted. Thus, this constellation of residues was highly selected for binding the Tax peptide, despite the fact that the residues were present in different CDR loops. The other positions (CDR1α 26, 28, 30 and CDR3α 98) in the degenerate library were biased toward particular residues, but variability at these positions suggested that different solutions for binding Tax/HLA-A2 existed.
Following selection with MART1/HLA-A2, five colonies were sequenced and analyzed. The clones all bound to MART1/HLA-2, but only three of them showed specificity for MART1 (i.e., did not bind to Tax or WT1) (Fig. 4.9b, center panels, and data not shown), whereas the other two also bound to Tax and WT1 (data not shown). Sequencing revealed that the three MART1-specific clones all had the same sequence (clones RD2-MART1-S3-1, -2, -3; called RD2-MART1), whereas the two clones that bound to MART1, Tax, and WT1 differed substantially from this sequence, but were similar to each other (clones RD2-MART1-S3-4 and -5) (Fig. 4.2). Interestingly, unlike the Tax-selected clones, all of the MART1-selected clones contained the four CDR3β residues (AGGR) of the wild-type A6 TCR at TCRβ positions 99-102.

The MART1-specific clones contained residues S26, H28, and Q30 in CDR1α; residues L100 and W101 in CDR3α; and residue M98 in CDR3β. Each of these selected residues was distinctly different from the Tax-specific clones, except for the tryptophan in CDR3α (W101). This finding supports the view that all of these residues contributed either directly or indirectly to the peptide specificity of the TCRs. The MART1-specific clones also contained two mutations, to serine and proline at positions 33 and 39, in framework region 2 (FR2) of the Vα chain that were likely incorporated through PCR errors (Fig. 4.2). The presence of a proline in framework regions has been observed in other TCR engineering studies39,41,71 and may be involved in stabilizing the V domains, thereby enhancing yeast cell surface levels.

The two clones that cross-reacted with all three peptide/HLA-A2 complexes, RD2-MART1-S3-4 and -5, shared the same residues at CDR1α S28, CDR3α R100, and
CDR3α S101 but differed at CDR1α 26, CDR1α 30, and CDR3β 98. One of the clones also contained a single-site mutation to a proline at CDR1α 25.

Following selection with WT1/HLA-A2, six colonies were sequenced and analyzed. All six clones bound to all three peptide ligands, WT1, Tax, and MART1 (Fig. 4.9b, right panels), and thus showed a binding phenotype similar to the two cross-reactive MART1-selected clones (RD2-MART1-S3-4 and -5) (Fig. 4.2). Interestingly, the sequences of all six clones were unique (Fig. 4.2), but they also shared significant similarities to the two MART1-selected, cross-reactive clones. The eight cross-reactive clones contained the CDR1α S28, CDR3α R100, and CDR3α S101 residues, suggesting that these were important in binding to the pep/HLA-A2 complexes regardless of the peptides. Three of the WT1-selected clones also contained the proline mutation at position 25 of CDR1α, and an aromatic residue at the adjacent amino acid, CDR1α 26. This pair of residues may be important in configuring the TCR to bind to the ligands, again independent of the peptide. Finally, one of the clones (RD2-WT1-S3-5) contained the CDR3β residues 99-102 (MSAQ) of the high-affinity Tax-specific TCR A6-X15, suggesting that it was not absolutely critical to have the potentially more flexible CDR3β residues of the wild-type TCR A6 (i.e. AGGR) to generate cross-reactivity. However, the cross-reactive clones did contain residues with smaller side chains at position 98 of CDR3β, compared to the leucine, tryptophan, or methionine found with the Tax- or MART1-specific clones.
**Attempted affinity maturation of cross-reactive RD2 library isolates**

Following the third selection of the RD2 library with WT1/HLA-A2, two additional selections were performed in attempts to reduce the number of cross-reactive clones allowing for a population of WT1/HLA-A2 specific clones to emerge, if present. A negative magnetic selection was performed with Tax/HLA-A2 followed by an additional positive selection with WT1/HLA-A2. Clones isolated from these two additional selection steps were all cross-reactive with the three pepMHC complexes tested (Tax/HLA-A2, MART1/HLA-A2, and WT1/HLA-A2) and contained scTCR sequences that resembled the cross-reactive clones examined after the third selection of the RD2 library (Fig. 4.2 and data not shown).

A further attempt was made to isolate WT1/HLA-A2-specific clones by generating a degenerate library in the CDR3β positions 98-102 using cross-reactive clones as a template, including two clones isolated from the 3rd sort of the RD2 library (RD2-WT1-S3-1 and RD2-WT1-S3-5) and one clone isolated following the 5th sort of the RD2 library (RD2-WT1-S5-2) (Fig. 4.11). Two negative selections were performed with Tax/HLA-A2, followed by two positive selections with MART1/HLA-A2 or WT1/HLA-A2 and one last negative selection with Tax/HLA-A2. All full-length clones isolated showed similar cross-reactivity to the template clones, staining positively for all three pepMHC complexes tested (Tax/HLA-A2, MART1/HLA-A2, and WT1/HLA-A2), although selected CDR3β residues varied (Fig. 4.11 and data not shown).

As a final attempt to engineer specificity among cross-reactive RD2 variants, two additional libraries were generated with degeneracies (NNK) in either the CDR1α or
CDR3α loops at positions selected in the original RD2 library that could account for cross-reactivity: positions D27 and G29 or positions S99 and W100, respectively (residue based on wild-type sequence) (Fig. 4.12 and Fig. 4.13). Templates used for the library included RD2-WT1 isolated clones used for the CDR3β library (RD2-WT1-S3-1, RD2-WT1-S3-5, and RD2-WT1-S5-2) as well as the non-truncated cross-reactive clones isolated from the selections of the RD2-WT1-CDR3β library with MART1 and WT1/HLA-A2. As before, two negative selections were performed with Tax/HLA-A2. Following the negative selections, both the unselected library and the negatively selected populations were subjected to two positive selections with either MART1/HLA-A2 or WT1/HLA-A2. Similarly, all clones isolated after the final selections were cross-reactive independent of selecting ligand (MART1/HLA-A2 or WT1/HLA-A2) or whether or not they underwent negative selections (data not shown). Sequencing of isolated clones following final selections revealed the strong bias for template amino acids at these positions, although some structurally similar variations occurred, e.g. F27α in RD2-WT1-CDR1/3α-MART1-S4-1 and 6, or W27α in RD2-WT1-CDR1/3α-WT1-S4-3; T29α in RD2-WT1-CDR1/3α-MART1-S4-1, RD2-WT1-CDR1/3α-WT1-S2-4, RD2-WT1-CDR1/3α-WT1-S4-3 and 5 (Fig. 4.12 and Fig. 4.13).

**Additional scaffold library design and selection attempts**

In addition to the RD1 and RD2 libraries described, an intermediary library, called RD1.5, was generated and selected, but did not yield clones with redirected specificity. The RD1.5 library was designed with five degenerate (NNS) positions: TCRα Q30, T98,
D99, and S100 and TCRβ L98 (Fig. 4.14). The TCRβ residues at position 99-102 were restricted to the wild-type sequence, AGGR. Dynamics studies have shown that the CDR3β loop of A6 is highly flexible and the presence of small residues such as alanine and glycine were predicted to reduce potential steric hindrance when bulkier residues were selected at the adjacent L98β position\textsuperscript{72}. Because structural models suggested that the arginine at position 27 in CDR1α could cause potential steric and/or electrostatic clashes if bulkier residues were selected residues at nearby degenerate positions, it was mutated to alanine. An alanine scanning study of a panel of high-affinity scTCRs including A6-X15 did not show a significant loss of binding when the arginine at position 27 of TCRα was mutated to alanine (discussed in chapter 2)\textsuperscript{23}.

The RD1.5 library was screened with six pepMHC ligands in four FACS selections: Tax/HLA-A2 (LLFGYPVYV; cognate), MART1/HLA-A2 (ELAGiGILTV; non-cognate), WT1 (RMFPNAPYL; non-cognate), Survivin (LTLGEFLKL; non-cognate), preproinsulin (ALWGPDPAAA; non-cognate), and NYESO-Val (SLLMWITNV; non-cognate)/HLA-A2. Clones isolated following the forth FACS selections did not show redirected specificity to non-cognate antigens, although staining suggested that several clones with positive staining were non-specifically binding to the IgG F(ab’)\textsubscript{2} AlexaFluor 647 secondary antibody through progressive sorts (data not shown).

Additionally, an attempt to isolate additional clones from the three libraries described was performed by combining the RD1, RD1.5, and RD2 libraries. In order to exclude clones which bound to the cognate antigen Tax/HLA-A2, two negative magnetic selections were performed prior to selecting with the following pepMHC antigens for 2-3
positive magnetic selections: WT1 (RMFPNAPYL; non-cognate), SL9/HIVgag (SLYNTVATL; non-cognate), Survivin-T2M (LMLGEFLKL; non-cognate), NYESO-Val (SLLMWITNV; non-cognate), preproinsulin (ALWGPDPAAA; non-cognate), MDM2 (VLFYLGQY; non-cognate), HBV Env (FLLTRILTI; non-cognate)/HLA-A2, and Tel1p (MLWGYLQYV; cognate). No additional clones with redirected specificities were isolated in this sorting progression as well. Control studies suggested that the high concentration of DMSO required to maintain insoluble peptides in solution may have negatively impacted yeast cell viability and/or the integrity of the peptide/MHC complexes throughout the sorting progression (data not shown).

**Site-directed mutagenesis of RD scaffold variants at position 98β**

Examination of the sequences of isolated RD variants suggested that TCRβ position 98 might be important in the specificity of the isolated scaffold variants, as all Tax-specific variants in this study selected the wild-type leucine at CDR3β position 98 whereas MART1-specific variants selected an alternate residue at this position (Trp in RD1 and Met in RD2). In order to assess whether this residue was pivotal in the specificity switch, converting A6-X15 into a MART1-specific TCR, this position was reverted to leucine in the MART1-specific scaffold variants (W98L for RD1-MART1 and RD1-MART1^HIGH, and M98L for RD2-MART1). Conversely, the RD1-Tax-1 clone (identical to A6-X15) was mutated to the residues selected in the MART1-specific variants (L98W and L98M). The yeast-displayed mutants were stained with various concentrations of peptide/HLA-A2 tetramers and analyzed by flow cytometry (Fig. 4.15).
The results showed that the residue selected at position 98 was critical for binding. The only exceptions were that: 1) the L98M mutation in A6-X15 resulted in binding to Tax/HLA-A2, but at a reduced level (Fig. 4.15a), and 2) the M98L reversion mutation of RD2-MART1 maintained specific binding to MART1. In no case was the specificity of the original TCR reverted to high-affinity binding of the alternative peptide, even at concentrations of pep/HLA-A2 tetramers that were 20-fold above detectable levels with the original clone. We conclude that residues at position 98 were important for high-affinity binding and specificity of the Tax or MART1 complexes (except for the RD2-MART1 clone), but this mutation alone was not responsible for the switch in peptide specificity. Consistent with this, the MART1-specific TCR DMF5 has a leucine at position β98, but mutation of this position to Trp enhanced affinity by > 3-fold28.

To further examine the role of A6 residue L98β in specificity for Tax/HLA-A2, Rosetta sequence tolerance algorithms were used to determine if residues other than the wild-type leucine had a significant probability of tolerance at the A6-c134:Tax/HLA-A2 interface (Fig. 4.16). The results showed that leucine was by far the most tolerated amino acid (predicted frequency of 90-100%), whereas all other amino acids combined yielded a frequency of less than 10%.

Binding studies of soluble RD1 scaffold variants

To further examine the binding properties of selected, peptide-specific TCR clones, the single-chain genes encoding RD1-Tax-1 (identical to A6-X15), RD1-MART1, and RD1-MART1^{HIGH} were cloned into an E. coli expression vector and proteins were
refolded from inclusion bodies. Soluble scTCRs were examined for binding to antigen presenting cell line T2 cells loaded with peptides, and/or by surface plasmon resonance (SPR). The binding of soluble RD1-MART1 and RD1-MART1\textsuperscript{HIGH} to MART1/HLA-A2 complexes on T2 was measured using biotinylated single-chain TCRs (scTCR genes were cloned with the C-terminal AviTag, and purified proteins were biotinylated \textit{in vitro} with BirA enzyme). Peptide-loaded T2 cells were incubated with various concentrations of biotinylated TCRs, followed by washing and incubated with SA-PE. The RD1-MART1 clone yielded positive staining above 500 nM, whereas the RD1-MART\textsuperscript{HIGH} TCR yielded positive staining at low nanomolar concentrations, with half-maximum staining at 30 nM. No staining was observed with Tax/HLA-A2 even at the highest concentration of TCRs (Fig. 4.17).

SPR was performed by flowing single-chain TCR proteins over immobilized pepMHC complexes or flowing pepMHC over immobilized scTCR. Similar to previous studies with the A6-X15 scTCR\textsuperscript{36}, the RD1-Tax-1 TCR exhibited a K\textsubscript{D} value of 84 nM for Tax/HLA-A2 based on equilibrium measurements (Table 4.1). The first generation MART1-specific TCR, RD1-MART1, exhibited a K\textsubscript{D} value of 3.1 \textmu M based on equilibrium measurements. The affinity-matured TCR RD1-MART1\textsuperscript{HIGH} exhibited a K\textsubscript{D} value of 68 nM based on kinetic measurements and 247 nM based on equilibrium measurements. An equilibrium titration of RD1-MART1 and RD1-MART1\textsuperscript{HIGH} with MART1/HLA-A2 or negative controls is shown in Fig. 4.18.

Yeast display, flow cytometry-based titrations with ligands have now been used in various studies as a method for examining binding affinities\textsuperscript{73,74}. The results of SPR
studies were consistent with various experiments in which monomers or multimers of the pep/HLA-A2 complexes were used to stain the yeast-displayed forms of these TCRs and half-maximal binding concentrations were determined (Table 4.1). Specificity of the yeast-displayed scTCR variants for their respective peptides was verified, as even the highest concentrations of pepMHC multimers did not bind to the null pepMHC ligands.

*Specificity analysis of A6-X15 and RD1-MART1 by molecular dynamics simulations*

In order to gain further insight into the underlying molecular mechanism of differential peptide specificity, comparative molecular dynamics (MD) simulations were performed on two complexes, A6:Tax/HLA-A2 and RD1-MART1:MART1/HLA-A2. In order to determine if MD was a valid method to analyze possible mechanisms of specificity of the RD1 variants, two sets of validation simulations were performed. First, we determined whether MD simulations would correctly predict the backbone and side chain orientations of the CDR3β loop of high-affinity TCR A6-c134 derived from A6. For this, the A6 wild-type crystal structure (PDB: 1AO7) was mutated to the CDR3β loop of A6-c134 (i.e. positions 99-102 mutated from AGGR to MSAQ). Following mutagenesis, the structure of the A6 wild-type with ‘MSAQ’ was subjected to a 40 ns simulation. The resultant conformations were overlaid with the experimentally characterized A6-c134 high-affinity crystal structure (PDB: 4FTV) and root mean square deviations (RMSDs) were calculated (Fig. 4.19). Among the configurations sampled during the simulation, there was significant CDR3β loop-overlap between simulated orientations and the A6-c134 crystal structure (RMSD < 1.5 Å for 44.3% of the trajectory) (Fig. 4.19).
In a second validation experiment, we determined if MD simulations could accurately predict the insertion of the side chain of residue F100β in the DMF5 TCR (PDB: 3QDG)\textsuperscript{6}. In the crystal structure, this residue inserts into a hydrophobic pocket formed between the HLA-A2 α1 helix and the MART1 peptide. In this MD simulation, the conformation of F100β was initially manipulated to move this residue out of the pocket (180° rotation of χ1 dihedral angle and a 90° rotation for χ2 dihedral angle prior the start of the simulation). Residue F100β was selected due to its predicted key interaction with MART1/HLA-A2 and its similar proposed mechanism of binding for the CDR3 tryptophans (W98β - 3HG1/Mel5-orientation or W100α - 3QDG/DMF5-orientation) of RD1-MART1 (Fig. 4.20 and see below). Within 0.1 ns into the simulation, the F100β reinserts into the binding pocket of MART1/HLA-A2 and remains in this position for the remainder of the simulation (4.3 ns). This rapid reorganization suggests that the F100β side-chain insertion is a critical feature of the interaction between DMF5 and MART1/HLA-A2. The consistency between the results of these two test simulations and the prior structural studies, as well as the success of earlier MD simulations in replicating structural and dynamic properties of TCRs and their complexes\textsuperscript{72,75} help reassure the validity of MD in exploring the determinants of specificity.

For MD simulations of RD1-MART1:MART1/HLA-A2, four different initial models were constructed based on the A6-c134:Tax/HLA-A2 orientation (PDB: 4FTV) as well as three different orientations observed for decameric MART1/HLA-A2 in complex with different MART1-specific TCRs (DMF5, DMF4, and Mel5; PDBs: 3QDG, 3QDM, and 3HG1, respectively)\textsuperscript{6,7,34} (see Materials & Methods for details). Two distinct peptide:TCR
interaction patterns were observed for A6 and RD1-MART1, which could account for their differential peptide binding specificity and affinity. Key interactions in the A6:Tax/HLA-A2 complex identified by screening for hydrogen bonding and electrostatic interactions included three hydrogen bonds, namely, $Y_5^{\text{Tax}}$-$R_95^{\beta}$, $Y_5^{\text{Tax}}$-$S_31^{\alpha}$, and $Y_8^{\text{Tax}}$-$E_30^{\beta}$ (Fig. 4.21a, and Fig. 4.22). A recent mutational study suggested that A6 interactions with $Y_8^{\text{Tax}}$ provided significant binding energy$^{16}$. Consistent with this, the L98$^{\beta}$ mutations in the present study likely influenced a van der Waals interaction between $Y_8^{\text{Tax}}$ and L98$^{\beta}$. Our simulations also identified a key hydrogen bond between $Y_8^{\text{Tax}}$ and E30$^{\beta}$.

The MD simulations also identified A6-c134:HLA-A2 interactions involving a salt bridge between D99$^{\alpha}$ and R65 of HLA-A2. Additionally, a salt bridge between D99$^{\alpha}$ and K66 of HLA-A2 occasionally formed during the simulations, with the orientation of the D99$^{\alpha}$ side chain being further stabilized by the hydroxyl group of T98$^{\alpha}$ (Fig. 4.22). These contacts likely play a role in further enhancing TCR:HLA-A2 interactions (Fig. 4.22). The importance of T98$^{\alpha}$ and D99$^{\alpha}$ in A6 TCR recognition has recently been established by mutational analysis$^{16}$. Interestingly, the selection of T98$^{\alpha}$ and D99$^{\alpha}$ by Tax/HLA-A2, but not by MART1/HLA-A2 (where K98$^{\alpha}$ and Y99$^{\alpha}$ were selected), also indicates that the peptide has a strong influence on these TCR:HLA-A2 interactions.

In contrast to the A6-c134:Tax/HLA-A2 complex, the major stabilizing interaction in RD1-MART1 complexes appeared to be the insertion of a tryptophan side chain from RD1-MART1 ($W_{101}^{\alpha}$ in the 3QDG/DMF5-orientation or $W_{98}^{\beta}$ in the 3HG1/Mel5-orientation) into the extra space provided by the highly curved topology of the MART1
peptide (Fig. 4.21b-d). This insertion of a tryptophan side chain was not observed in the 4FTV/A6-c134 or 3QDM/DMF4 orientations (Fig. 4.23), a finding also supported by calculations of the solvent accessible surface area (SASA) (Fig. 4.24). This suggests that the 4FTV/A6-c134 and 3QDM/DMF4 orientations are unlikely to represent optimally bound configurations for the complex, and that the more likely mode of interaction is similar to those observed in the 3QDG/DMF5 or 3HG1/Mel5 crystal structures. The exclusion of a 3QDM/DMF4-like orientation is further consistent with the structural data, as the DMF4 TCR does not use the Va2 gene segment. The 4FTV/A6-c134 and 3QDM/DMF4 orientations were also characterized by a counter-clockwise rotation (when viewed from the top of the TCR) with regard to the other two orientations. The orientation in 3QDG/DMF5 (but not 3HG1/Mel5) allows the establishment of two salt bridges, K55α with E154MHC of HLA-A2 and D56β with R65MHC of HLA-A2, which have interaction probabilities of 51.1% and 97.5%, respectively (Fig. 4.21e and Fig. 4.22). Thus, although both 3QDG/DMF5 and 3HG1/Mel5 orientations show tryptophan insertion, only the former orientation simultaneously maintains both of these inter-domain salt-bridges, making the 3QDG/DMF5 orientation the more likely representative of the complex (Fig. 4.21d,e). These CDR2 interactions (K55α/E154MHC and D56β/R65MHC) have been described in several TCR complexes15,76, and they help facilitate the conserved TCR diagonal orientation observed for most TCR:pepMHC complexes4,76.

It is interesting to note that the DMF5:MART1/HLA-A2 structure (PDB: 3QDG), similar to the modeled RD1-MART1:MART1/HLA-A2 complex, also contains the Va2
region, MART1, and HLA-A2. Although the TCR (DMF5) in the 3QDG/DMF5 structure does not contain a tryptophan in either CDR3, a phenylalanine at position 100 in the CDR3β inserts into the same area of the MART1/HLA-A2 ligand as predicted by the MD simulations (Fig. 4.25). Rosetta sequence tolerance algorithms were also used to determine if this phenylalanine was highly restricted in this position. This result showed a strong preference for tyrosine and phenylalanine (Fig. 4.16). Although the salt bridges described above are not present, R65\textsubscript{MHC} of HLA-A2 plays a key role, as it does in most other complexes\textsuperscript{6,76}.

Finally, we examined what would happen if MART1 were replaced by Tax in the MD simulations of the RD1-MART1:MART1/HLA-A2 complexes. In this simulation, the tryptophan insertion could not be established due to the different backbone topology of the Tax peptide (Fig. 4.21f). In addition, neither of the salt-bridges mentioned above formed, and the triple hydrogen bonding pattern observed in the A6-Tax/HLA-A2 complex was absent in the MD simulation of the RD1-MART1:Tax/HLA-A2 complex. Additionally, when MART1 is replaced by Tax in a model of the complex with A6-c134, A6 was found in the simulation to lose not only the triple hydrogen bonds, but also the D98α-K66\textsubscript{MHC} salt bridge. Collectively, these effects likely account for the differential affinity of A6 and RD1-MART1 for MART1/HLA-A2 and Tax/HLA-A2.

**Discussion**

The peripheral T cell repertoire is shaped by positive and negative selection in the thymus, whereby T cells with TCRs that do not bind to self-pepMHC, or with TCRs
that bind too strongly, are deleted\textsuperscript{1,2}. The wild-type TCRs that have been isolated and characterized biochemically and structurally have been derived from T cells that have made it through these stringent \textit{in vivo} selection processes. In this study, we created repertoires of TCRs in yeast display libraries that could be selected for pepMHC binding \textit{in vitro}, without the \textit{in vivo} “filters” that are involved in thymic or peripheral T cell processes. We believe that this \textit{in vitro} selection approach can provide additional information about the fundamental basis of pepMHC specificity of TCRs. In addition, the strategy represents a high-throughput system to generate specific TCR leads against the thousands of potential targets represented by viral or cancer peptides, without the need to isolate T cell clones for each one.

Our primary goal in the present study was not to affinity-mature a wild-type TCR against its cognate antigen\textsuperscript{18-23,25-28}, but to use directed evolution to isolate TCRs with novel specificities against non-cognate antigens. We chose the human TCR A6 as an initial scaffold based on the wealth of biochemical and structural information available for this TCR, and previous findings that it is amenable to yeast display and directed evolution\textsuperscript{36}. The A6 TCR recognizes at least three distinct HLA-A2-restricted ligands, Tax (LLFGYPVYV) derived from HTLV-1, and two structural mimics of Tax called Tel1p (MLWGYLQYV) and HuD (LGYGFVNYI)\textsuperscript{6,15,77}. Studies of A6 binding to these and various other mutant Tax peptides have shown significant plasticity in CDR3 loops to accommodate a variety of substitutions in the Tax structure\textsuperscript{62,72,78}. The use of an \textit{in vitro} directed evolution approach as described here is also founded on the now well
established ability of a single TCR to cross-react with multiple pepMHC ligands\textsuperscript{79}, a feature that has been attributed to a variety of mechanisms (reviewed in\textsuperscript{13,80,81}).

Two different libraries of the A6 TCR resulted in TCR variants that bound to the non-cognate antigen MART1/HLA-A2. To the best of our knowledge this is the first report of “switching” the specificity of a TCR to a completely different peptide by directed evolution. The MART1-specific TCRs from both of the libraries contained similar mutations within the CDR3 loops. In RD1-MART1, a tryptophan (98\(\beta\)) replaced the leucine of the wild-type A6 TCR, and mutagenesis results showed that the W98\(\beta\) played a key role in binding. In RD2-MART1, a tryptophan at position 101 of the CDR3\(\alpha\) was selected, and it is possible that it functions in a manner similar to the W98\(\beta\) (see discussion of MD simulations, below). In RD1-MART1, a lysine was selected at position 98 of the CDR3\(\alpha\), whereas in RD1-MART1, an arginine was selected at position 102 of CDR3\(\beta\). Strikingly, the affinity-matured variant of RD1-MART1 (RD1-MART1\textsuperscript{HIGH}) evolved four residues in CDR3\(\beta\) (MAGG, 99-102) that were also selected, in a one-amino-acid register shift, in the RD2-MART1 clone (MAGG, 98-101). While a structure of these mutants will be required to fully understand the underlying molecular basis of MART1 specificity, it is likely that it involves the unique contribution of these residues from both CDR3 loops.

The two different libraries reported here also yielded higher-affinity TCRs for binding to the Tax/HLA-A2 complex. Here, as with the MART1-specific clones, there was strong selection for specific residues in the CDR3\(\beta\), including 4 residues at positions 99-102 (MSAQ), which have been identified previously as yielding a high-
affinity phenotype. However, in the context of these residues, there was also strong selective pressure for Tax binding evidenced in the preference for residues in CDR3α. For example, four CDR3α residues within the two different libraries (T98 and D99 in RD1, and S100 and W101 in RD2) were highly restricted in Tax selections, and these are the same residues found in the wild-type A6 TCR. This finding supports the idea that both CDR3 loops operate in concert to provide specificity, and higher affinity, for Tax.

Interestingly, WT1-specific TCRs were not isolated, despite the presence at position 8 in both Tax and WT1 of a tyrosine that is predicted to be a key residue for binding by A616. The absence of TCR variants that bound WT1 among the A6 libraries again supports the view that multiple regions across the TCR interface are involved in conferring specificity (i.e., not only those near the Y8 in these two peptides). It is possible that alternative libraries, with degeneracies in other CDR residues, could yield solutions to WT1 binding.

The basis of the specificity switch from Tax to MART1 was also investigated by MD simulations of known structures, or of models of the selected TCRs docked onto structures of these pep/HLA-A2 ligands. MD has previously been used to assess the role of conformational heterogeneity in TCR/pepMHC interactions72,82. To validate the use of MD simulations to probe functionally relevant TCR:pepMHC interactions, three analyses were performed. First, MD was used to assess the CDR3β side chain orientation sampled for a modeled A6-c134 TCR using the wild-type A6 crystal structure as a template for computational mutagenesis. The resulting structures demonstrate that the orientation observed in the published A6-c134 crystal structure is adopted in 44.3%
of the trajectory (RMSD < 1.5 Å). Second, we showed the ability of MD simulations to correctly predict hydrophobic insertion of a TCR CDR3β side chain in an interaction with MART1/HLA-A2 through the F100β position of the DMF5 TCR. Finally, an MD simulation was performed using the A6:Tax/HLA-A2 structure to verify known interactions. The resultant simulation was in agreement with previously reported results using a variety of biophysical and mutational approaches. The simulation results indicate that major interactions between the A6 TCR and the Tax/HLA-A2 complex involved Tax residues Y5 and Y8 and HLA-A2 residue R65, along with key TCR residues, S31α, T98α, D99α, E30β, and L98β. Interestingly, although CDR3α residues T98 and D99 interacted with R65/K66 of HLA-A2 and they were highly conserved in selection with Tax/HLA-A2, this restriction was completely dependent on the peptide. Thus, selection of the RD1 library with MART1 yielded only CDR3α residues K98 and Y99, which must provide very different modes of interaction with the MART1/HLA-A2 complex.

The MD simulations of the RD1-MART1 TCR revealed a quite striking mode of interaction that could account for a substantial binding difference between MART1 and Tax complexes. A tryptophan in either CDR3α or CDR3β was inserted into the space created by the curved position of the MART1 backbone, providing significant hydrophobic stabilization. This positioning also oriented the outside of the periphery of the TCR binding surface, through the two CDR2 loops, to form two salt bridges (one with each HLA-A2 helix). The specificity of RD1-MART1 for MART1 was accounted for in part by the inability of Tax to accommodate this tryptophan conformation and thus to
allow optimal orientation and interaction between the TCR and the Tax/HLA-A2 complex. Further MD simulations and structural studies with the RD1-MART1 TCR and RD2-MART1 isolated here are in progress.

One of the libraries (RD2) yielded TCR variants that were cross-reactive with different pep/HLA-A2 ligands. These cross-reactive clones were selected with either non-cognate ligand, MART1/HLA-A2 or WT1/HLA-A2. The sequences of the cross-reactive clones revealed diversity, but highly restricted residues were selected at particular positions of CDR1\(\alpha\), CDR3\(\alpha\), and CDR3\(\beta\). The consensus sequence of these clones, as represented by clone RD2-WT1, involved the following five residues: I26\(\alpha\), S28\(\alpha\), R100\(\alpha\), S101\(\alpha\), and V98\(\beta\). To gain insight into the basis of the binding of these cross-reactive TCRs, and their peptide independence, Rosetta Backrub models were generated for various complexes of this canonical mutant TCR, based on the A6 structure. The wild-type A6:Tax/HLA-A2 structure was compared to the models of the RD2-WT1 TCR docked onto Tax/HLA-A2, MART1/HLA-A2 or WT1/HLA-A2 (Fig. 4.26a). None of the five residues in A6 were less than 3.2\(\AA\) from the nearest HLA-A2 residues, although several were involved in contacts with Tax. In striking contrast, all five of the RD2-WT1 residues were predicted to be positioned within 3\(\AA\) of the HLA helices, including the following paired residues [TCR residue:HLA-A2 residue(s)]: I26\(\alpha\):E58, S28\(\alpha\):Y59/W167, R100\(\alpha\):A69/Q72, S101\(\alpha\):R65, and V98\(\beta\):T73 (Fig. 4.26b,c).

In order to further explore the basis of cross-reactivity in this class of isolates as well as to attempt to reverse engineer cross-reactive clones from the RD2-WT1 selections for specificity for either WT1/HLA-A2 or MART1/HLA-A2, CDR3\(\beta\) libraries
were generated using several cross-reactive RD2-WT1 clones as templates. Selected clones maintained cross-reactivity, although there was a strong preference for aromatic residues (i.e. tyrosine, phenylalanine or tryptophan) surrounded by smaller side chains (e.g. glycine, alanine, leucine, serine) and occasionally arginine. The generation of the CDR1α and CDR3α libraries from both these and the original RD2-WT1-selected clones showed a strong preference at RD2-degenerate positions (i.e. TCRα 27, 29, 99, and 100) for the residues selected in the cross-reactive class of RD2 isolates. This suggests that these positions in particular are likely to account for the cross-reactivity of this class of TCRs, perhaps through non-specific interactions with the HLA-A2 α1 and α2 helices predicted to be within proximity in Rosetta models (Fig. 4.26). This also could account for the inability to reverse engineer cross-reactive clones from the RD2-WT1 selections for specificity for either WT1/HLA-A2 or MART1/HLA-A2.

The highly restricted nature of each of these residues in the cross-reactive TCRs, across three different loops, suggests that they act in concert to contribute to binding of the HLA-A2 molecule. Importantly, all five residues are predicted to be at a sufficient distance from each peptide to avoid steric clashes that would prevent binding. T cells that expressed TCRs such as these RD2 clones would likely have been negatively selected in the thymus. In fact, relevant to this, transgenic mice containing a single peptide/MHC as selecting ligand yielded T cells with TCRs that exhibited similar cross-reactive behavior. In addition, the highly restricted sequences of these TCRs, and those selected with specificity for Tax or MART1, are similar to the dominance of some TCRs that arise from positive selection on self-peptides in the thymus.
In summary, using a single TCR scaffold we have shown that it is possible to generate novel TCRs against non-cognate pep/MHC ligands. To improve the likelihood of isolating specific TCRs de novo against other peptide/HLA-A2 complexes (such as WT1), several approaches can be applied to the design of additional libraries, including the use of a collection of TCR templates with different $V_\alpha 2$ and $V_\beta$ regions, degeneracies at other CDR positions and the use of synthetic CDR3 loops with varying amino acid lengths. In addition, it may be possible to optimize negative selections to remove cross-reactive TCRs such as RD2-WT1 variants that have a basal affinity for most peptide/HLA-A2 complexes. These libraries could be engineered for specificity by diversifying CDR residues that could provide specific binding energy with peptide residues unique to the peptide of interest.

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**Figures**

**Figure 4.1** Structures of Tax, MART1 and WT1 peptides and design of the first generation TCR scaffold library RD1.  
(a) Structure of the HLA-A2-bound Tax peptide (LLFGYPVYV) (PDB: 1DUZ)
85, black. (b) Structural alignment of the HLA-A2-bound decamer MART1 peptide (ELAGIGILTV) (PDB: 1JF1)
86, magenta, and the HLA-A2-bound nonamer MART1 peptide (PDB: 2GUO)
88, blue. (c) Structure of the HLA-A2-bound WT1 peptide (RMFPNAPYL) (PDB: 3HPJ)
87, cyan. (d) Five residues, Q30α, T98α, D99α, L98β, and A101β (G in the wild-type A6), generated as degenerate codons in the RD1 library as found in the structure of the A6-c134:Tax/HLA-A2 complex (PDB: 4FTV)
34, green. A6-c134 contains the same CDRβ sequence at positions 99-102 as the single-chain A6-X15 that was used as the RD1 library template. The Tax peptide is shown in black, the MART1 decamer peptide from the aligned Mel5:MART1/HLA-A2 structure (PDB: 3HG1)
7 is shown in magenta, and the WT1 peptide from the aligned WT1/HLA-A2 structure (PDB: 3HPJ)
87 is shown in cyan.
Figure 4.2 | Amino acid sequences from CDR1α, CDR3α, CDR3β and framework region 2α (FR2α) of various RD-derived TCR clones. Residues of the A6 wild-type TCR \(^8\) and the high-affinity variant A6-X15 \(^6\) are shown. Positions of degeneracy in the RD1 and RD2 libraries are highlighted, where X represents any amino acid (based on NNS or NNK nucleic acid composition in yellow and cyan, respectively). RD2 position 30 indicated with “T” in magenta indicates a binary position where either wild-type Q or A was present in the library. RD2 positions 99-102 in CDR3β indicated by four consecutive “*”s indicate a binary string where the four adjacent residues were either A6 wild-type (AAGR) or A6-X15 (MSAQ). PCR-based mutations in selected TCR mutants are highlighted in orange. Mutants isolated from the RD1 selections are shown (RD1-Tax-1, RD1-Tax-2, and RD1-MART1) as well as the affinity-matured RD1-MART1 variant, RD1-MART1\(^{\text{HIGH}}\) (selected residues from affinity maturation are highlighted in gray). Sequences of other RD1 isolates are shown in Figs. 4.4 and 4.7). Variants selected from the RD2 library following the third sorts are shown. RD2-Tax-S3-1 to S3-6 clones were specific for Tax, and RD2-MART1-S3-1 to S3-3 clones were identical and specific for MART1 (also called RD2-MART1). The RD2-MART1-S3-4 and S3-5 clones listed separately showed binding to all three peptide complexes (i.e. Tax, MART1, and WT1), as did all six clones isolated with WT1/HLA-A2 (RD2-WT1-S3-1 to S3-6). Residue numbering is consistent with the crystal structures of A6:Tax/HLA-A2 (PDB: 1AO7) \(^8\) and A6-c134:Tax/HLA-A2 (PDB: 4FTV) \(^34\). TCR variants’ names are colored according to their determined specificities: Tax/HLA-A2 (red), MART1/HLA-A2 (blue), and cross-reactive (green).
Figure 4.3 | Flow cytometry histograms of the selections of the RD1 library with cognate antigen Tax/HLA-A2 and non-cognate antigen MART1/HLA-A2, and affinity maturation of RD1-MART1. (a) The RD1 library was sorted sequentially with Tax/HLA-A2-Ig dimer. Aliquots of yeast cells after each sort were then incubated with 100 nM Tax/HLA-A2-Ig dimer followed by APC-conjugated goat anti-mouse secondary antibody. (b) The RD1 library was sorted sequentially with MART1 (ELAGIGILTV)/HLA-A2-Ig dimer for a total of five sorts. During the 3rd sort, yeast cells were also stained with chicken anti-c-myc antibody, goat anti-chicken IgY alexa 488 secondary antibody and double positives were isolated in order to exclude truncated clones (not shown). Aliquots of yeast cells after each sort were then incubated with 100 nM MART1 (ELAGIGILTV)/HLA-A2-Ig dimer followed by APC-conjugated goat anti-mouse secondary antibody. (c) RD1-MART1 was used as a template for affinity maturation libraries in CDR3 loops, and combined libraries were sorted sequentially with MART1/(ELAGIGILTV)/HLA-A2-Ig dimer for a total of two sorts. Aliquots of yeast cells after each sort were then incubated with 50 nM MART1 (ELAGIGILTV)/HLA-A2-Ig dimer, followed by APC-conjugated goat anti-mouse secondary antibody. Data is representative of 2 experiments with similar results.
**Figure 4.4** | Sequence alignment of clones isolated from the RD1 library, following each round of selection with Tax/HLA-A2. The sequences of the A6-X15 template and RD1 library are shown, and degenerate residues in the RD1 library are highlighted in yellow. Clones isolated from each of the rounds of sorting are indicated by S1 to S4. The two Tax-specific variants referenced in Fig. 4.2, RD1-Tax-1 and RD1-Tax-2, are repeated in the bottom two rows. Residue numbering is consistent with the crystal structures of A6:Tax/HLA-A2 (PDB: 1AO7)\(^8\) and A6-c134:Tax/HLA-A2 (PDB: 4FTV)\(^34\).
Figure 4.5 | Binding of selected TCR clones from the RD1 library to Tax/HLA-A2 or MART1/HLA-A2. Following four sorts of the RD1 library with Tax (LLFGYPVYV)/HLA-A2 and five sorts of the RD1 library with MART1 (ELAGIGILTV)/HLA-A2 as shown in (Fig. 4.3a,b), individual yeast clones were analyzed for peptide/HLA-A2 binding. (a) The RD1-Tax-1 clone isolated from Tax selections (top panels), that was identical in amino acid sequence to A6-X15, and the RD1-MART1 clone isolated from MART1 selections (bottom panels), were stained with various concentrations of the Tax/HLA-A2-Ig dimer (left panels) or MART1/HLA-A2-Ig dimer (right panels) at the indicated concentrations. Gray filled histograms were yeast cells stained with secondary antibody only. (b) Plot of the mean fluorescence intensity (MFI) from staining RD1-Tax-1 and RD1-MART1 with various concentrations of Tax/HLA-A2-Ig dimers (left) and MART1 (ELAGIGILTV)/HLA-A2-Ig dimers (right) at 4 to 500 nM. The half maximal effective concentration (EC_{50}) determined by nonlinear regression analysis is indicated. (c) The RD1-MART1^{HIGH} clone, isolated by affinity maturation of the RD1-MART1 clone, was stained with various concentrations of Tax/HLA-A2 monomers (left panel) or MART1/HLA-A2 monomers (right panel) at the indicated concentrations. Gray filled histograms are yeast cells stained with secondary antibody only. Data is representative of 4 experiments with similar results.
Figure 4.6 | Sequence alignment of clones isolated from the RD1 library following each round of selection with MART1/HLA-A2. The sequences of the A6-X15 template and RD1 library are shown, and residues made degenerate in the RD1 library are highlighted in yellow. Clones isolated from each round of sorting are indicated by S1 to S5. The MART1-specific variant referenced in Fig. 4.2, RD1-MART1, is repeated in the bottom row. Residue numbering is consistent with the crystal structures of A6:Tax/HLA-A2 (PDB: 1AO7) and A6-c134:Tax/HLA-A2 (PDB: 4FTV).
Figure 4.7 I Sequence alignment of clones isolated from the RD1-MART1 CDR3 affinity maturation library following two selections with MART1/HLA-A2. Three five-codon (NNK) libraries, CDR3α, CDR3β-1, and CDR3β-2, shown in gray were generated and combined to create the RD1-MART1 CDR3 affinity-maturation library. Following two selections with MART1/HLA-A2 dimer (200 nM and 1 nM), 10 clones were isolated and sequenced (shown as RD1-MART1-CDR3 #1-10). Eight clones were parental, one clone called RD1-MART1-CDR3-8 contained a Q81K PCR-based framework mutation in the α-domain (shown in orange), and one clone, called RD1-MART1<sup><sub>HIGH</sub></sup>, was derived from the CDR3α library and had three mutations that differed from the parental sequence (S100A, Q102G, and P103V; shown in gray). Residue numbering is consistent with the crystal structures of A6:Tax/HLA-A2 (PDB: 1AO7)<sup>8</sup> and A6-c134:Tax/HLA-A2 (PDB: 4FTV)<sup>34</sup>.
Figure 4.8 Staining of the RD1-MART1$^{\text{HIGH}}$ clone to assess cross reactivity with other HLA-A2 restricted peptides. The yeast-displayed RD1-MART1$^{\text{HIGH}}$ clone was stained with 10 nM MART1/HLA-A2-Ig dimer (a) or 500 nM Tax/HLA-A2-Ig dimer (b), WT1/HLA-A2-Ig dimer (c), or Survivin/HLA-A2-Ig dimer (d), followed by APC-conjugated goat anti-mouse secondary antibody. Gray indicated histograms of yeast stained with secondary reagent only. Data is representative of 2 experiments with similar results.
Figure 4.9 | Binding of selected TCR clones from the RD2 library to Tax/HLA-A2 or MART1/HLA-A2. (a) Residues of the A6 TCR used in the design of the RD2 library. The structure of the aligned wild-type A6:Tax/HLA-A2 (PDB: 1AO7) and A6-c134:Tax/HLA-A2 (PDB: 4FTV), that contained the CDR sequences used as templates for the RD2 library, are shown with degenerate positions at D26α, G28α, S100α, W101α, and L98β (green). The binary residues (Gln or Thr) at position Q30α is shown in red, and the backbone of the binary string of wild-type “AGGR” or high-affinity “MSAQ” in positions 99-102β is shown in yellow. The Tax peptide is in black, the MART1-peptide from the aligned Mel5:MART1/HLA-A2 structure (PDB: 3HG1) is in green, and the WT1 peptide from the aligned WT1/HLA-A2 structure (PDB: 3HPJ) is in cyan. (b) A Tax-selected clone (RD2-Tax-S3-1) (left panels), a MART1-selected clone (RD2-MART1) (middle panels), and a WT1-selected clone (RD2-WT1-1) isolated from the RD2 library were each stained with various concentrations of the indicated peptide/HLA-A2-Ig dimers. Gray filled histograms were yeast cells stained with secondary antibody only. Data is representative of 3 experiments with similar results.
Figure 4.10 | Selection of a second A6 library called RD2. Two sequential magnetic bead selections of the RD2 library were performed following incubation with 1 μM Tax/HLA-A2 or 5 μM MART1 or WT1/HLA-A2 UV-exchanged monomers and streptavidin MACS beads (Miltenyi Biotec). A third selection was performed with FACS following incubation with 1 nM Tax/HLA-A2-Ig dimer or 100 nM MART1 or WT1/HLA-A2-Ig dimer, respectively. Aliquots of yeast cells after each selection were incubated with 50 nM selecting/HLA-A2-Ig dimer, APC-conjugated goat anti-mouse secondary antibody. (a) Flow cytometry histograms of the RD2 library after sorting with the cognate antigen, Tax (LLFGYPVYV)/HLA-A2. Gray indicates histograms of yeast cells stained with secondary antibody only. (b) Flow cytometry histograms of the RD2 library after sorting with the non-cognate antigen, MART1 (ELAGIGILTV)/HLA-A2. (c) Flow cytometry histograms of the RD2 library after sorting with the non-cognate antigen, WT1 (RMFPNAPYL)/HLA-A2. Data is representative of 2 experiments with similar results.
Figure 4.11 | Sequence alignment of clones isolated from the RD2-WT1-CDR3β library. The five-codon (NNK) library generated with three RD2 cross-reactive template clones (RD2-WT1-S3-1, RD2-WT1-S3-5, and RD2-WT1-S5-2) is shown in gray. Degenerate residues are indicated with “X.” The five clones isolated from magnetic selections with either MART1/HLA-A2 or WT1/HLA-A2 following 5 sorts (negative sorts 1, 2, and 5 used 5 μM Tax/HLA-A2; positive sorts 3 and 4 used 5 μM selecting ligand) are shown (#1-5). An underscore (_) indicates the selection of a stop codon. Residue numbering is consistent with the crystal structures of A6:Tax/HLA-A2 (PDB: 1AO7) and A6-c134:Tax/HLA-A2 (PDB: 4FTV).
Figure 4.12 | Sequence alignment of clones isolated from the combined RD2-WT1-CDR1α and 3α libraries with MART1. The two-codon (NNK) libraries generated from templates including three RD2 cross-reactive clones (RD2-WT1-S3-1, RD2-WT1-S3-5, and RD2-WT1-S5-2) and non-truncated RD2-CDR3β library isolates (MART1/WT1-S5 #1-5) are shown. Degenerate residues (NNK) are indicated with “X,” and positions that varied in the library based on multiple templates are highlighted in blue. The RD2-WT1-CDR1α and RD2-WT1-CDR3α libraries were combined in equal cell numbers prior to selections. The first group of clones shown (S2) were isolated following two positive magnetic selections with 5 μM MART1/HLA-A2. The second group of clones shown (S4) were first subjected to two negative selections with 5 μM Tax/HLA-A2 before two positive selections with 5 μM MART1/HLA-A2. PCR-based mutations are highlighted in orange. Residue numbering is consistent with the crystal structures of A6:Tax/HLA-A2 (PDB: 1AO7)\(^3\) and A6-c134:Tax/HLA-A2 (PDB: 4FTV)\(^3\).

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Figure 4.12 | Sequence alignment of clones isolated from the combined RD2-WT1-CDR1α and 3α libraries with MART1. The two-codon (NNK) libraries generated from templates including three RD2 cross-reactive clones (RD2-WT1-S3-1, RD2-WT1-S3-5, and RD2-WT1-S5-2) and non-truncated RD2-CDR3β library isolates (MART1/WT1-S5 #1-5) are shown. Degenerate residues (NNK) are indicated with “X,” and positions that varied in the library based on multiple templates are highlighted in blue. The RD2-WT1-CDR1α and RD2-WT1-CDR3α libraries were combined in equal cell numbers prior to selections. The first group of clones shown (S2) were isolated following two positive magnetic selections with 5 μM MART1/HLA-A2. The second group of clones shown (S4) were first subjected to two negative selections with 5 μM Tax/HLA-A2 before two positive selections with 5 μM MART1/HLA-A2. PCR-based mutations are highlighted in orange. Residue numbering is consistent with the crystal structures of A6:Tax/HLA-A2 (PDB: 1AO7)\(^3\) and A6-c134:Tax/HLA-A2 (PDB: 4FTV)\(^3\).
Figure 4.13 | Sequence alignment of clones isolated from the combined RD2-WT1-CDR1α and 3α libraries with WT1. The two-codon (NNK) libraries generated from templates including three RD2 cross-reactive clones (RD2-WT1-S3-1, RD2-WT1-S3-5, and RD2-WT1-S5-2) and non-truncated RD2-CDR3β library isolates (MART1/WT1-S5 #1-5) are shown. Degenerate residues (NNK) are indicated with “X,” and positions that varied in the library based on multiple templates are highlighted in blue. The RD2-WT1-CDR1α and RD2-WT1-CDR3α libraries were combined in equal cell numbers prior to selections. The first group of clones shown (S2) were isolated following two positive magnetic selections with 5 μM WT1/HLA-A2. The second group of clones shown (S4) were first subjected to two negative selections with 5 μM Tax/HLA-A2 before two positive selections with 5 μM WT1/HLA-A2. PCR-based mutations are highlighted in orange. Residue numbering is consistent with the crystal structures of A6:Tax/HLA-A2 (PDB: 1AO7) and A6-c134:Tax/HLA-A2 (PDB: 4FTV)3d.
Figure 4.14 | Sequence alignment showing the RD1 and RD2 libraries aligned with the RD1.5 library. Residues of A6 wild-type TCR\(^8\) and the high-affinity variant A6-X15\(^{36}\) are shown. Positions of degeneracy in the RD1.5 library are highlighted in gray, where X represents any amino acid (based on NNS nucleic acid composition). RD1.5 position 27\(\alpha\) which was mutated to alanine is shown in orange. No clones with redirected specificities were isolated from the RD1.5 library. Residue numbering is consistent with the crystal structures of A6:Tax/HLA-A2 (PDB: 1AO7)\(^8\) and A6-c134:Tax/HLA-A2 (PDB: 4FTV)\(^{34}\).
Figure 4.15 | Binding analysis of various position 98β mutants of selected TCRs. Binding titrations of the indicated mutants of A6-X15 (a), RD1-MART1 (b), RD1-MART1^{HIGH} (c), and RD2-MART1 (d). Mutants were stained with 12.3 nM, 37.0 nM, 111 nM, 333 nM, and 1 μM Tax and MART/HLA-A2 PE-conjugated streptavidin tetramers. Normalized percent max mean fluorescence intensity (MFI) is plotted against peptide/HLA-A2 tetramer concentration. This experiment was also performed with pepMHC-Ig dimers with similar results (data not shown).
Predicted Rank

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| DMF5 100β |
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| 5 | N |
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| 8 | D |
| 9 | A |
| 10 | L |
| 11 | H |
| 12 | E |
| 13 | V |
| 14 | Q |
| 15 | G |
| 16 | R |
| 17 | K |
| 18 | P |
| 19 | C |

Figure 4.16 | Prediction of TCR reaction specificity using Rosetta sequence tolerance algorithms. Crystal structures of the A6-c134 (PDB: 4FTV) (a) and DMF5 (PDB: 3QDG) (b) were used as input for Rosetta sequence tolerance algorithms \(^{49,50}\). Structural models of the predicted 10 lowest energy level configurations were made and mutated to the other possible 19 amino acids. The average predicted frequencies of each amino acid at the indicated TCRβ positions in the ensemble are presented as a ranked list with ranges specified by color. The top 5 residues are indicated by a dotted line. According to Smith and Kortemme\(^{49,50}\), 42-82% of amino acids from experimental phage display data occur above the dashed line in experimental validation datasets.
Figure 4.17 | Binding of soluble TCRs (RD1-MART1 and RD1-MART1<sup>HIGH</sup>) to human T2 cells loaded with Tax or MART1. T2 cells were loaded with Tax or MART1, followed by incubation with biotin-labeled RD1-MART1 (left) or RD1-MART1<sup>HIGH</sup> (right) scTCR at the indicated concentrations. The shaded gray histograms show staining with the null Tax-loaded T2 cells at the maximum concentration of TCR for each experiment (2 µM and 5 µM, respectively). Data is representative of 2 experiments with similar results.
Figure 4.18 | Equilibrium Binding of RD1-MART1 and RD1-MART1\textsuperscript{HIGH}. SPR binding of immobilized RD1-MART1 (a) and RD1-MART1\textsuperscript{HIGH} (b) scTCRs with MART1/HLA-A2 (selecting, non-cognate; black), Tax/HLA-A2 (non-selecting, cognate; purple), and gp100 (non-selecting, non-cognate; red). Data at each concentration are representative of two injections. Averages and standard deviations of all SPR experiments (including those in the reverse orientation) are shown in Table 4.1.
Figure 4.19 | MD simulation of the modeled A6-c134 CDR3β loop. (a) Root mean square deviation (RMSD) of the CDR3β modeled positions 99-102 (based on the A6 wild-type crystal structure, PDB: 1AO7) over 40 ns. The A6-c134 crystal structure (4FTV) was employed as the reference. An RMSD of <1.5 Å suggests a good sidechain overlap with respect to the crystal structure at the indicated positions. Raw data is shown in lighter color, and the block-averaged data are shown by the darker line. (b) Overlay of the backbone configurations of the A6-wt ‘MSAQ’ trajectory are shown in red, with the starting A6 wild-type configuration (green) and A6-c134 crystal structure (blue). (c) Sidechain orientations of an A6-wt ‘MSAQ’ trajectory configuration (RMSD of 0.6 Å) compared to their orientations in the A6-c134 crystal structure (4FTV).
Figure 4.20 | MD of DMF5 TCR Residue F100β. (a) Snapshots of DMF5 residue F100β from the simulations compared with the original crystal structure conformation. The conformation of F100β from the crystal is shown in red, the starting conformation is shown in yellow, the final conformation is shown in cyan, and the intermediates are also shown in cyan in stick representation. (b) Root mean square deviation (RMSD) of the heavy atoms of the F100β sidechain from the crystal structure orientation. (c) χ1 and χ2 dihedral angles of F100β. For both (b) and (c) raw data is shown in lighter color, and the block-averaged data are shown by darker lines.
Figure 4.21 I Snapshots of TCR:peptide/HLA-A2 interactions based on MD simulations. (a) Specific hydrogen bonding and salt-bridge interactions are shown for A6-c134:Tax/HLA-A2. (b) A superposition of A6-c134:Tax/HLA-A2 and RD1-MART1:MART1/HLA-A2 (3QDG/DMF5-orientation), highlighting the extra space created by the curvature of MART1 but not by Tax. (c) Insertion of tryptophan W101α of RD1-MART1 into the interface between MART1 and HLA-A2 in the 3QDG/DMF5-orientation. (d) Insertion of tryptophan W98β of RD1-MART1 into the interface between MART1 and HLA-A2 in the 3HG1/Mel5-orientation. (e) Salt bridge interactions that occur in the 3QDG/DMF5-orientation of the RD1-MART1:MART1/HLA-A2 complex. (f) The absence of tryptophan insertion in RD1-MART1 when Tax replaces MART1 in the HLA-A2 complex (4FTV/A6-c134-orientation).
Figure 4.22 | MD predicted interactions between TCR and peptide or MHC. The colors indicate the probability of strong interaction, which is measured by the fraction of time residues interact with a distance less than 3.5 Å during the simulation. The size of each marker is also proportional to the interaction probability. The optimal interaction distance during interactions is indicated on the y-axis.
Figure 4.23 I MD of interactions of alternative RD1-MART1 orientations. No significant hydrophobic interactions exist between W98β and W101α and MART1 peptide when RD1-MART1 is in 4FTV/A6-c134-orientation (a) or 3QDM/DMF4-orientation (b).
Figure 4.24 | MD of solvent accessible surface area in modeled complexes. Hydrophobic interactions between W98β (a,c) or W101α (b,d) and the peptide-MHC complex, indicated by solvent accessible surface area (relative). The A6-c134 (c,d) and RD1-MART1 (a,b) TCRs are abbreviated as A6 and RD1, respectively.
Figure 4.25 | Overlay of RD1-MART1 models and the DMF TCR. Superposition of the DMF TCR with RD1-MART1 in the 3QDG/DMF5-orientation and RD1-MART1 in the 3HG1/Mel5-orientation, using MHC as an alignment reference. The key aromatic residue at position 98β (for the 3HG1/Mel5-orientation) and 101α (for the 3QDG/DMF5-orientation) are highlighted in red and green, respectively. The aromatic F100β of the aligned DMF5 TCR crystal structure is in yellow. The MART1 peptide in each structure or model is shown with matching color.
Figure 4.26 | Modeling of RD2-WT1 that yielded cross-reactivity with non-selecting ligands. Rosetta Backrub flexible backbone modeling algorithms were used to model the RD2-WT1 mutations (D26αI, red; G28αS, orange; S100αR, yellow; W101αS, green; and L98βV, blue) into the wild-type A6 crystal structure (PDB: 1AO7). HLA-A2 residues within 3.2 Å of RD2-WT1 residues are labeled in italics and shown as sticks (gray) (a) The wild-type A6 crystal structure (PDB: 1AO7) showing RD2-WT1 positions prior to mutation. (b) The A6 crystal structure (PDB: 1AO7) with mutated RD2-WT1 residues. Tax peptide is shown in black. (c) The A6 crystal structure (PDB: 1AO7) with mutated RD2-WT1 residues and mutated WT1 peptide in the HLA-A2 binding groove (cyan).
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<th>$K_D$ equilibrium (kinetic)</th>
<th>$EC_{50}$ HLA-A2 monomer</th>
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Table 4.1 | Binding properties of RD1 library-derived TCR clones. SPR experiments were performed at 25°C with peptide/HLA-A2 monomers immobilized on a sensor chip and soluble scTCR flowed over in solution. For one replicate with RD1-MART1 and RD1-MART1\textsuperscript{HIGH} scTCRs, the orientation was reversed such that soluble scTCR was immobilized on a sensor chip and soluble pepMHC was flowed over in solution (curves shown in Fig. 4.1). The MART1 peptide used for these experiments was the anchor modified decamer MART1 variant (i.e. MART1\textsubscript{26–35 A27L}: ELAGIGILTV). For equilibrium SPR values, n=4 for RD1-MART1 and n=3 for RD1-MART1\textsuperscript{HIGH}. For kinetic SPR values, n=1 for A6-X15 and RD1-MART1\textsuperscript{HIGH}. $EC_{50}$ values for titrations on the surface of yeast with soluble peptide/HLA-A2 monomers and Ig dimers are reported with n=1. The A6-X15 TCR (sequence identical to RD1-Tax1) value was previously measured to be 53.4 nM by SPR\textsuperscript{36}. T1-S18.45 is a MART1-specific TCR, previously engineered for higher affinity with a measured affinity of 45 nM\textsuperscript{23}. Typically $EC_{50}$ values are higher than SPR values at affinities ($K_D$) >100 nM due to wash steps prior to flow cytometry analysis; A possible explanation for the discrepancy of RD1-MART1\textsuperscript{HIGH} is that the scTCR protein was less stable in solution at high concentrations.
References


Haidar, J. N. *et al.* Structure-based design of a T-cell receptor leads to nearly 100-fold improvement in binding affinity for pepMHC. *Proteins* 74, 948-960 (2009).


79 Mason, D. A very high level of crossreactivity is an essential feature of the T-cell receptor. Immunology today 19, 395-404 (1998).
APPENDIX A

GENES, LIBRARIES & PRIMERS

A.1 Synthesized gene constructs

1.1 T1 scTv gene (from INRi-T1 T cell clone)

Source: Genscript

TCR α-chain usage: Vα2

TCB β-chain usage: Vβ16

Notes: codon optimized for yeast, synthesized with F49Sα mutation for stability

N-terminal flanking sequence: 5’ – ggtggttctgctagc – 3’

C-terminal flanking sequence: 5’ – ctcgagatctgt – 3’

scTv construct (Vβ-Linker-Vα):

```
gaagcttgttgtacctcaattcccatctcatgcattagaaaggtgctagaccgtgact
E A G V T Q F P S H S V I E K G Q T V T
ctgcgctgtgtgctgccgatttctggtatgataatatgtgtagtgtagttatggt
L R C D P I S G H D N L Y W Y R R V M G
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K E I K F L L H F V K E S K Q D E S G M
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P N N R F L A E R T G G T Y S T L K V Q
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V E Q Y F G P G T R L T V E D L K N G
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E Q N S G P L S V P E G A I A S L N C T
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Y S D R G S Q S F F W Y R Q Y S G K S P
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E L I M S I Y S N G D K E D G R F T A Q
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L N K A S Q Y V S L L I R D S Q P S D S
```
1.2R1 scTv gene (from INRi T cell clone)

Source: Genscript
TCR α-chain usage: Vα2
TCB β-chain usage: Vβ14
Notes: codon optimized for yeast

N-terminal flanking sequence: 5’ – ggtgttctgcctagc – 3’
C-terminal flanking sequence: 5’ – ctgcagatctgt – 3’

scTv construct (Vβ-Linker-Vα):

gcacttatctgtgcgggtaatgataacgcagctcgctctgatgattggcgatgtaccagc
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gccacttatctgtgcgggtaatgataacgcagctcgctctgatgattggcgatgtaccagc
AT YLCAVNDA RLFMDGTQ
tggttgtgaaaccgaatatttaatag
L V V K P N I – –
1.3 Survivin71 scTv gene

Source: Genscript
TCR α–chain usage: Vα2
TCB β–chain usage: Vβ20
Notes: codon optimized for yeast, synthesized with F49Sα mutation for stability

N-terminal flanking sequence: 5’ – ggtggttctgtagc – 3’
C-terminal flanking sequence: 5’ – ctcgagatctgt – 3’

scTv construct (Vβ-Linker-Vα):

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1.4WT1-P20 scTv gene

**Source:** Genscript  
**TCR α-chain usage:** Vα2  
**TCB β-chain usage:** Vβ3  
**Notes:** codon optimized for yeast, added a c-myc tag (EQKLISEEDL)

**N-terminal flanking sequence:** 5’ – ggtggttctgctagc – 3’  
**C-terminal flanking sequence:** 5’ – ctcgagatctgtgcg – 3’

**scTv construct (Vβ-Linker-Vα):**

```
gatgttaaagtgacacaatcttcacgtttatctggtgaaaaagacggtgaaaaaggtcatttt  
DVKVTSRRYLVKRTGEKVFTtagaatgtgttaagatatgatatgctagc
LECVQDMDFNMFYWQRDPGttaggcttgccctgtctatattttcctctagatgttaagaggaagaaagcgatc
LGLRLYFSDVDVKMKKEKGDICCagaaggctatctctgtgtaaaagaagagatgtctcctagctttgtcttggatcc
PEGYSVSRKKERFSLILESgcctctaccaaatcagacttctatgtacctggtgtcagctcacatcaaccacagacttt
ASTNQTSMYLCASTSHQPQFGgcgatggcactagactgacatctttagagatatttgaaacaaggtatgtgctgtatgct
GDGTLLSDILEDNLKGSADDAaagaaaaagatgctgtcattagaaagatggcaaatctcgaagaaaggtgaaaccatgtgt
KADAKKDGKSQKEVEQNSGccttggcctggtccgggagaagccacattgctgactatcctgacacatctcctgatc
PLSVPEGAIASLNCTYSDRGtcacaaaagctttttctgtatatagacagtactctgtgcaaatcaccggaattatatgac
SQSFFWRQYSISGKSPELIMSatctacagtaatggcgataaagaagatggccgctttcactgcccaactgacacaagaacgtca
IYSNGDKEGRFTAQLNKScagtatgtcagctgttaatccctgtgatttacacacatgtgactccgctacatctgtgc
QYVSLLIRDSQPSDSATYLCgcagtctaggaagggcagaaatggtgctttgctgcctccgacagttgatatgtctg
AVLEGQKLFLFRGTMKLKVDAacatgcaaaaaattactcagtgaaggaagatttgataataan
NIEQLISEEDL--
```
1.5 WT1-P22 scTv gene

Source: Genscript
TCR α-chain usage: Vα2
TCB β-chain usage: Vβ3
Notes: codon optimized for yeast, added a c-myc tag (EQKLISEEDL)

N-terminal flanking sequence: 5' – ggtggtttctgtagc – 3'
C-terminal flanking sequence: 5' – catatgcgcagatctgtgcgt – 3'

scTv construct (Vβ-Linker-Vα):

gatgttaagtgacacaatccttcacgttattttgttaaaaagacggtgaaaagatctttt
DVKTQSSRYLVTGKEQVT
ctggatgtgtttcaagatatggatcatgaaacatgttctgtacagacaggatctggc
LECVQDMHDHNMFYWRYQQDPG
	tgggtctgctgcctgtatctttttcatacgatgtaaagatgaaagaaaagcgcata
	cGLRLIFYSDVKMKEKGDI
ccagaggtttatacttttcgtgtaaagaaagagacgtttcagtttgtcctgaaagc
PEGYSVSREKKERFSLIES

gctagtaccaaatcacaacttccatgtacctgtgtcgaacagttccatcaacgaacagttt
ASTNQTSMYLCASSSINEQF

ttcggccccaggtaacaggtttctgtgagatctgttctgcctgat
gatggtttctgtgagatctgttctgcctgat

C-terminal flanking sequence:

5' – catatgcgcagatctgtgcgt – 3'

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1.6 HLA-A2 α1α2 module

Source: Genscript
Notes: codon optimized for yeast, added a c-myc tag (EQKLISEEDL)

N-terminal flanking sequence: 5’ – tctgctagc – 3’
C-terminal flanking sequence: 5’ – tagtagctcgagatctga – 3’

HLA-A2 module construct (α1-α2):

```
GGCTCTATTCTATGCCTACTTTTACTTCGTCAGTCGCCCTGGCCGTTGGTAACC
GSRSMMRFFTSVSRPGREGEP
CGCTTTATTGTGTGGTTATGTGGATGATACACAAATTGTATTAGATTGGATTCTGAGCT
RFIAYVDFTQQFVRFDSDA
GCACTACAAGAACAGCAGCACATTCACAGACACACCGTTGGATCTGGCAGC
ASQRMERPAPWIEQEPEYW
GATGCGAACACGCCAAGTCAGCGACACATACAGACACACCGTTGGATCTGGCAGC
DGERTKVKAHSQTHRVDLGT
TTAAGGGCTATTACAATCAGGAGCTGGTTATCAGATCACGTCGTAGCTCAG
LRYYNQSEAGSHTVQRMYG
TTGATGGTGGCTCGACTGCGCTTTCTGCTGGTTATCAGGAATATGCGTCGGTG
CDVGSDFRWFLRLGYHQYAYDG
AAAGACTACATCGCCTGTAAGGAGATTTCGCGTCGTGGACCGACAGACATGGCTGA
KDYIALKEDLRSWTAADMAACAGAACACTAAACATAAGGGAGGGCGCGACCGTTGGATCAGAAATACGTGCA
QTTKHKWEAAHVAEQLRAYL
GAAAGCCTTTGCCTGGATGGCTCGTGGATTAGATACCTTAGAACAAGGCGAAGGACATTCA
EGTCVIEWSLRLRRYLENKETLQ
CGCACCAGAACAATTGATTAGCGAGAAGCCTTG
RTEQKLISEEDL
```
A.2 Synthesized libraries

2.1 Rational design library 1 (RD1)

Source: Genscript
Scaffold template: A6-X15 scTv
TCR α-chain usage: Vα2
TCB β-chain usage: Vβ13
Notes: codon optimized for yeast, added a c-myc tag (EQKLISEEDL)

N-terminal flanking sequence: 5’ – tctgctagc – 3’
C-terminal flanking sequence: 5’ – ctcgagatctga – 3’

Degeneracies: NNS degenerate codons are indicated in bold and underlined.

scTv library (Vβ-Linker-Vα):

aatgctgtgtataacacaaacgcGccaaatctccaggttttaagacagggccatccatgacgc
NAGVTVTPKFQVLKGTQSMT
nttcaatgtgtcaggaacatgaaacatkgaataatatgccatgtaccgtcaggatccgggtLQCAQDMHYMAYRQDPG
atggctttaagattgattcactattccagttagtggttggcatcactgatcaaggtgacgtagMGRLRLIHYSVGVGITDQGDV
ccggatggctacaagatctctgcctaaaccactgaagattttccgctgcgtctgttaagcPDCGYKVSRSSTTEDFPRLRLS
Gctgcaccttcctcaacacattcctgtatatctctccgcttcgggttnnsatgtcggtnns
AAPSVGYFCASRPBXMSX
CagcctgcaactctgtactttgttgcagccacactgaactgacgcctgaactgattatataatQPELYFGPTRLTVDLAIN
ggtagccgcgtagacgcGcGcCaaaggtgccaaagtgctaaaagtaaggggaaaGSAADDACKDDAKKGDGSQKE
GtggaaacaaatattcgtgctccactgtcagtccgggaagcgccatcgcgtcttttaactgtVEQNSGPGPLSVPEGAIASLNCAcatattccagataaggtagcnnscttttcttttgggatcgcagttacacgGcCaaaagtTYSDDRGSXSFFFWYRSYSGKS
Cctgatattaattatgtccatctaactgagttgagaagatggggcctcaaggcctgctctctacgct
PELIIMSAYSNGDKEDGRFTA
CaacctgaaacagcagggcagctagtattattttgctgtactccagcctctccgatQLNKASQYVSSLIRDSQPSD
TcggccacacactctgtgcgggttagcnnsnnsntcgtgagtttaacactgacatgtgctcgSATYLCAVTXSXSWGKLQFGAGGcaccaggttggctcactccagacagaaacaaatgtgatcctgagaagatattataAA
GTQVVVTDPDEQKLISEEEDL−tga
2.2 Rational design library 1.5 (RD1.5)
Source: Genscript
Scaffold template: A6-wt scTv
TCR α−chain usage: Vα2
TCB β−chain usage: Vβ13
Notes: codon optimized for yeast, added a c-myc tag (EQKLISEEDL), introduced an
R27Aα mutation (italicized and bolded below)
N-terminal flanking sequence:
5’ –
ggcagccccataaacacacagtatgtttttaaggacaatagctcgacgattgaaggtagata
cccatacgacgttccagactacgctctgcaggctagtggtggtggtggttctggtggtggtg
gttctggtggtggtggttctgctagc
– 3’
C-terminal flanking sequence:
5’ –
ctcgagatctgttaacaacagtgtagatgtaacaaaatcgactttgttcccactgtactttt
agctcgtacaaaatacaatatacttttcatttctccgtaaacaacatgttttcccatgtaat
atccttttctatttttcgttccgttaccaactttacacatactttatatagctattcacttc
tatacactaaaaaactaagacaattttaattttgctgcctgccatatttcaatttgttataa
attcctataatttatcctattagtagctaaaaaaagatgaatgtgaatcgaatcctaagaga
attgagctccaattcgccctatagtgagtcgtatta
– 3’
Degeneracies: NNS degenerate codons are indicated in bold and underlined.
scTv library (Vβ-Linker-Vα):
aacgcgggtgttactcagacgccgaagttccaagttttgaagacaggccaaagcatgaca
N A G V T Q T P K F Q V L K T G Q S M T
ttacagtgcgctcaggatatgaatcacgaatatatggcttggtaccgccaagacccgggt
L Q C A Q D M N H E Y M A W Y R Q D P G
atgggcttacgtttgattcattatagcgttggtgtgggcatcaccgatcagggtgacgtc
M G L R L I H Y S V G V G I T D Q G D V
cctgatggctacaaagtaagcagaagtaccactgaagattttcctctgcgcctgttaagt
P D G Y K V S R S T T E D F P L R L L S
gctgcaccatctcaaacatcagtctatttttgtgcttctcgtcctggtnnsgcaggtggc
A A P S Q T S V Y F C A S R P G X A G G
agaccagaactgtacttcggtcctggtacgcgtctgacagtgacggaagatttgattaat
R P E L Y F G P G T R L T V T E D L I N
ggttctgccgatgacgcgaaaaaggacgccgcgaaaaaggatggcaaatcacaaaaggaa
G S A D D A K K D A A K K D G K S Q K E

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gttgaacaaatcctggtccattatcagtgcgggaagggctatcgcatttgaactgt
V E Q N S G P L S V P E G A I A S L N C
acctatcggatggcggttctnnstcaattttttctgtatcgtctcagatcagcggcabaagggt
ty s d a g s x s f f w y r q y s g k s
ccagaaatctgattatgtccatctactgaatggtcagcaggaagaagatggcgggtttatgcgcc
plemis y s n g d k e d g f t a
cattaaacagggcagagcagatgtcagtatttgctgttagtagagactctcacaaccatccgat
q l n k a s q y v s l l r d s q p s d
tcggtctaccacttctttgctgcagtaactnnnnnnnnntgggtaaactgcagttcccgtgctgc
g s a t y l c a v t x x x w g k l q f g a
ggcacccaggtcggtggtacgcctagtagaacaagaagcgtgatttccgagaagacttatgaga
g t q v v v t p d e q k l i s e e d l -
tga
-

2.3 Rational design library 2 (RD2)

Source: DNA2.0
Scaffold template: A6-wt and A6-X15 scT
TCR α-chain usage: Vα2
TCB β-chain usage: Vβ13
Notes: codon optimized for yeast, added a c-myc tag (EQKLISEEDL)

N-terminal flanking sequence:
5’ -
ggcagcccctataacacacaagatctgtatgtttaaagaaataagcagtttgaaggtagata
cccatagcagcttccagactacgtctgctcagggtagttgggtggtgtgggtttctggtgggtgtgtgtggtgtgttgtcgcagtgc
-
3’

C-terminal flanking sequence:
5’ -
cctgagatctgtttaaacaacacagtgtatgaataaaatcagacatgtttgtttccactgttatat
agctcgtacaacaataataatataactttttcatattttacttgacaacatggttttcctcatgtaat
tctcttttatattttttgtctgctcataactttacatattatagctatattccatctattatacaagacatttaaatctcattatctttagctagctaatataaaacagagatgtgtggtacaattcagataagcagagcctatagtgagttggtatttaa
-
3’

Degeneracies: NNK degenerate codons are indicated in bold and underlined; codon indicated by ‘^^^’ indicates a binary position where either T or Q can be selected; codons indicated by ‘***’ and ‘1234’ indicate a binary string where either the wild-type A6 (i.e. AGGR) or A6-X15 (i.e. MSAQ) CDR3β positions 99-102 can be selected.
scTv library (Vβ-Linker-Vα):

```
aacgcggaggtatacgcttcgaggtagttggtgggtgtgtgtgtgtgttct
NAGVYALQAAGSGGGSGGGSGGTGTTGTGTGTGTGTTCT
GGGSASNAGVTPFKPQV
aaaacaggcaagcatgacattacaatgatggctcagacatgaatcacaccaaatatggtct
KGTQSMTCQMDNHEYMA
ttgtaaacaggcaagattcaggatgtaggcctgaagatctatccattatctctgtggttcggt
WYQRDPGMGLRILHYSVGVG
atcacaagcagatggcttgccagcagcatgacaatttcggtctagcagatagctta
GSGSNAGVTQTPKFKQV
IITDQGDVPGYKVSSSTTED
ccctcttcgagattattgtccgagcagcactctgatcaataaggtatctggtccactacagaagat
FPRLRSSAAPSQTSTSVFCAS
agacctggcNNK************ccagaacctctacttccggccaggacagtagattaacc
RPPGΧ1234PELYFGPGTRLT
gttaccgggaagatttgatcaacgttgctcagcagcagtgctaaaggatcagcagccaaaaag
VTEDLINGSADDAKKDAAKKGacggaaaagtagtaaagaggtgcagaacactccggctcgactatcaggtccagaagggcgg
DGKSEQVEQNSGPSVPEGAA
atacctgtagttggaattggacattatcagttctgatccagcagcagcagtcctagcagttctgatcag
ΙAΣLNCΤYSXRXSΟΟΤSSFYW
caatattctgttaaaggccagacacttatattgctcaatctcagttgatcagcagagag
QYSGKSLPELIIMSIESNGDKEGatggctggtttcacagccagttgtagacagataagcctctagcagtttctattgtaaaaagcag
DGRFTΑQLNKASQYVSSLIR
atatgcactctctgactgcatatatagttgtgcgtacagacagatNNKNNKggc
DSQPΣDΣATYLCAVTDXG
aaattacataattcgggtcgctggccaggctgtgtagatcagttgtaaacactctgatattgacaaaaactg
KLQFGAGTQVVVPDIEEQKL
atctctggagaagagatctctgataaa
ISEQUEDL--
```
A.3 Primers for library synthesis, site-directed mutagenesis, and sub-cloning

All primers were obtained from Integrated DNA Technologies (IDT). Primers are listed by template in the order of date purchased.

Types of primers:
- 454 = Amplicon primers for 454 sequencing
- DL = Primers to generate degenerate libraries
  - N = Mix of A, T, C, and G
  - S = Mix of C and G
  - K = Mix of G and T
- SC = Sub-cloning
- SDM = Site-directed mutagenesis primers

3.1 Templates based on the 868 scTv and variants

<table>
<thead>
<tr>
<th>Template Clone(s)</th>
<th>Type</th>
<th>Date</th>
<th>Purpose</th>
<th>Primer Sequences</th>
</tr>
</thead>
</table>
| 868-Z11           | SDM  | 26 JUL 2011| Generate 868-Z11 | **FOR:** 5'-CTT CTT TGA ACT GTA CTT ACT CTG ATG CAG GTT CTC AAT CTT TCT TTT GGT -3'
|                   |      |            |                  | **REV:** 5'-CCA AAA GAA AGA TTG AGA ACC TGC ATC AGA GGT ACA GGT CAA AGA AG -3'      |
|                   |      |            |                  | **FOR:** 5'-CTG TAC TTA CTC TGA TAG AGG TTC TGC ATC TTT CTT TTG GTA CAG ACA ATA C -3'
|                   |      |            |                  | **REV:** 5'-GTA TTG TCT CTA CCA AAA GAA AGA TGC AGA ACC TCT ATC AGA GTA AGT ACA G -3' |
| 868-Z11           | SDM  | 26 JUL 2011| Generate 868-Z11 | **FOR:** 5'-CTC CAG AAT TGA TCA TGT CCA TCG CCT CTA ATG GTG ACA AAG AAG ATG -3'
|                   |      |            |                  | **REV:** 5'-CAT CTT CTT TGT CAC CAT TAG AGG CGA TGG ACA TGA TCA ATT C -3'          |
| 868-Z11           | SDM  | 26 JUL 2011| Generate 868-Z11 | **FOR:** 5'-TTC TTT GAA CTG TAC TTA CTC TGC TAC AGG TTC TCA ATC TTT CTT TTG -3'
|                   |      |            |                  | **REV:** 5'-AAA AGA ATT TAG GAG AAC CTC TAG CAG AGT AAC TAC GAT TCA AAG AA -3'  |
| 868-Z11           | SDM  | 28 JUN 2012| Generate 868-Z11 | **FOR:** 5'-TTT ACC ATG GGC AGC AGC CAT CAT CAT CAC AGC AGC GGC ACT CTG TTG GTG
|                   |      |            |                  | **REV:** 5'-AAA GAA ATG AGA ACC TCT ATA AGA GTA AGT ACA GGT CAA AGA AGG AG -3'  |
| 868c              | SC   | 22 MAR 2013| Sub-cloning into pET28 | **FOR:** 5'-TAT ACC ATG GGC AGC AGC CAT CAT CAT CAT CAC AGC AGC GGC ACT CTG TTG GTG
|                   |      |            |                  | **REV:** 5'-TTT GAA ATT CTT AGA TAT GTG GGG TAA CCA GCA GAG AGG TAC C -3'  |
3.2 Templates based on the A6 scTv and rational design library (RD1 and RD2) variants

<table>
<thead>
<tr>
<th>Template Clone(s)</th>
<th>Type</th>
<th>Date</th>
<th>Purpose</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>RD1 library</td>
<td>SC</td>
<td>22 JUN 2011</td>
<td>Add pCT302 overhangs to synthesized RD1 library</td>
<td>FOR: 5'- CAG GCT AGT GGT GGT GGT TCT GGT GGT GGT GGT GGT GGT GGT GGT GCT AGC AAT GCT GGT GTA ACA CAA ACG CCA A -3' REV: 5'- GGA ACA AAG TCG ATT TTG TTA CAT CTA AAC TGT TGT TAA CAG ATC TCG AGT CAT TAT AAA TCT TCT TCA GAG ATC -3'</td>
</tr>
<tr>
<td>A6-X15</td>
<td>SDM</td>
<td>26 JUL 2011</td>
<td>Generate A6-X15 R28Aα</td>
<td>FOR: 5'- GCT TCT TTG AAT TCT ACT TAC TCA GAT GCA GGC TCC CAA TCA TT -3' REV: 5'- AAT GAT TGG GAG CCT GCA TCT GAG TAA GTA CAG TTC AAA GAA GC -3'</td>
</tr>
<tr>
<td>A6-X15</td>
<td>SDM</td>
<td>26 JUL 2011</td>
<td>Generate A6-X15 Q31Aα</td>
<td>FOR: 5'- TAC TTA CTC AGA TAG AGG CTC CGC ATC ATT TTT CTG GTA TAG ACA G -3' REV: 5'- CTG TCT ATA CCA GAA AAA TGA TGC GGA GCC TCT ATC TGA ATG A -3'</td>
</tr>
<tr>
<td>A6-X15</td>
<td>SDM</td>
<td>26 JUL 2011</td>
<td>Generate A6-X15 Y51Aα</td>
<td>FOR: 5'- CCA GAA TTG ATG TCT ATC GCT AGC AAT GCT GGT GTA ACA CAA ACG CC -3' REV: 5'- TCC TCC TTA TCA CCG TTG CTA GCG ATG ATG ATC AAT TCT GGG CT -3'</td>
</tr>
<tr>
<td>A6-X15</td>
<td>SDM</td>
<td>26 JUL 2011</td>
<td>Generate A6-X15 S52Aα</td>
<td>FOR: 5'- AGC CCA GAA TTG ATG TCT ATC TAT GGC AAC GGT GAT AAG GA -3' REV: 5'- TCC TTA TCA CCG TTG GCA TAG ATA GAC ATG ATC AAT TCT GG -3'</td>
</tr>
<tr>
<td>A6-X15</td>
<td>SDM</td>
<td>28 JUN 2012</td>
<td>Generate A6-X15 D27Aα</td>
<td>FOR: 5'- CTT TGA ACT GTA CTT ACT CAG CTA GAG GCT CCC AAT CAT TTT T -3' REV: 5'- AAA AAT GAT TGG GAG CCT CTA GCT GAG TAA GTA CAG TTC AAA G -3'</td>
</tr>
<tr>
<td>A6-X15</td>
<td>SDM</td>
<td>28 JUN 2012</td>
<td>Generate A6-X15 D27Yα</td>
<td>FOR: 5'- CTT CTT TGA ACT GTA CTT ACT CAT ATA GAG GCT CCC AAT CAT TTT TC -3' REV: 5'- GAA AAA TGA TTG GGA GGC TCT ATA TGA GAT AGT ACA GTT CAA AGA AG -3'</td>
</tr>
<tr>
<td>A6-X15</td>
<td>SDM</td>
<td>28 JUN 2012</td>
<td>Generate A6-X15 D27Wα</td>
<td>FOR: 5'- TGC GAT TGC TTC TTT GAA CTG TAC TTA CTC ATG GAG AGG CTC CCA ATC AT -3' REV: 5'- ATG ATT GGG AGC CTC TCC ATG AGT AGT TCA AAG AAG CAA TCG CA -3'</td>
</tr>
<tr>
<td>RD1- MART1</td>
<td>SC</td>
<td>14 MAR 2012</td>
<td>Sub-cloning into pET28</td>
<td>FOR: 5'- TAT ACC ATG GGC AGC AGC CAT CAT CAT CAT CA CAC AGC AGC GGC CTG TG TGC CCG GCC AGC AGC ATG GCT GGT GTA ACA CAA ACG CC -3' REV: 5'- TTT AGA ATT CTT AGT CTG GAG TGA CCA CAA CCT GGG T -3'</td>
</tr>
<tr>
<td>Date</td>
<td>Library</td>
<td>FOR: (same as above from 14-MAR-2012)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>---------------</td>
<td>--------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>09 OCT 2012</td>
<td>Sub-cloning</td>
<td>(same as above from 14-MAR-2012)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>into pET28 with BirA tag</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
|            | REV: 5'- TTT AGA ATT CTT ATT CGT GCC ATT CGA TTT TCT GAG CCT CGA AGA TG TCA GAC CGC CAC CGT CTG GAG TGA CCA CAA CCT GGG T - 3' |}

<table>
<thead>
<tr>
<th>Date</th>
<th>Library</th>
<th>REV from O1 (to Splice4L): 5'- ACC CGG ACG GGA AGC GCA GAA ATA CAC TGA GGT TTG AGA AGG TGC AGC GCT TAA CAG ACG CAG CGG - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 DEC 2012</td>
<td>Attempt to generate the RD2 library with multiple primers and overlaps (i.e. O1, O2, &amp; O3); library ended up being synthesized by DNA2.0</td>
<td>FOR: (same as above from 14-MAR-2012)</td>
</tr>
<tr>
<td></td>
<td>REV from O1 to O2 (A6-X15): 5'- ACC TCA GTG TAT TTC TGC GCT TCC CGT CGG GGT NNS ATG TCG GCC CAG CCT GAA CTG TAC TTT GGT CCA GGC ACT AGA CTG - 3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FOR from O1 to O2 (A6-wt): 5'- ACC TCA GTG TAT TTC TGC GCT TCC CGT CGG GGT NNS ATG TCG GCC CAG CCT GAA CTG TAC TTT GGT CCA GGC ACT AGA CTG - 3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>REV from O2 to O1 (A6-wt &amp; A6-X15): 5'- TGA TAT TGT ACA GTT TAA AGA CGC GAT GGC GCC TTC CGG GAC TGA CAG TGG ACC AGA ATT TTG TTC CAC CAC CTC TTT CTT TTG GTA TCG CCA GTA CAG CCG C - 3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FOR from O2 to O3 (Q31α): 5'- CCC GGA AGG CGC CAT CGC GTC TTT AAA CTG TAC ATA TTC ANN SAG ANN SAG CCA GTC TTT TTG GTA TCG CCA GTA CAG CCG C - 3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FOR from O2 to O3 (T31α): 5'- CCC GGA AGG CGC CAT CGC GTC TTT AAA CTG TAC ATA TTC ANN SAG ANN SAG CAC CTC TTT TCT TTG GTA TCG CCA GTA CAG CCG C - 3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>REV from O3 to O2 (Q31/T31α): 5'- GTC GGT CGT AAC CGC GCA CAA GTA TGT GGC CGA ATC GGA AGG CTG GGA GTG AAT GGA CAG CAA AC ACT AAC ATA CTG GC - 3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FOR to O3 (to T7): 5'- TCG GCC ACA TAC TTG TGC GCG GTT AGC ACC GAC NNS NNS GGT AAA CTG CAA TTT GTG GCG GCC ACC CAG GTT GGA TGC G - 3'</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Date</th>
<th>Library</th>
<th>FOR: 5'- TCC GAT TCG GCC ACA TAC TTG TGC CGG GTT ACG NKN NKN NKN NKN NKN AAA CTG CAA TTT GTG GCG GCC ACC CAG GTT GTG G - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 FEB 2013</td>
<td>Generation of the RD1-MART1 CDR3α library</td>
<td>REV: 5'- CGT AAC CGC GCA CAA GTA TGT GGCC CGA ATC GGA AGG CTG GGA GTG AAT GGA CAG CAA ACT AAC ATA CTG GC - 3'</td>
</tr>
<tr>
<td></td>
<td>REV: 5'- ACC TCA GTG TAT TTC TGC GCT TCC CGT CGG NKN NKN NKN NKN NKN CAG CCT GAA CTG TAC TTT GTG CCA GGC ACT AGA C - 3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FOR: 5'- ACC TCA GTG TAT TTC TGC GCT TCC CGT CGG NKN NKN NKN NKN NKN CAG CCT GAA CTG TAC TTT GTG CCA GGC ACT AGA C - 3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>REV: 5'- CGG ACG GGA AGC GCA GAA ATA CAC TGA GGT TTG AGA AGG TGC AGC GCT TAA CAG ACG CAG CCG - 3'</td>
<td></td>
</tr>
<tr>
<td>Mutagenesis</td>
<td>SDM</td>
<td>Date</td>
</tr>
<tr>
<td>-------------</td>
<td>-----</td>
<td>----------</td>
</tr>
<tr>
<td>A6-X15</td>
<td>SDM</td>
<td>22 Mar 2013</td>
</tr>
<tr>
<td>A6-wt</td>
<td>SDM</td>
<td>22 Mar 2013</td>
</tr>
<tr>
<td>A6-X15</td>
<td>SDM</td>
<td>09 Aug 2013</td>
</tr>
<tr>
<td>RD1-MART1</td>
<td>SDM</td>
<td>09 Aug 2013</td>
</tr>
<tr>
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<td>06 Dec 2013</td>
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<td>A6-wt</td>
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<td>13 Feb 2014</td>
</tr>
<tr>
<td>A6-wt</td>
<td>A6-D27A</td>
<td>A6-R28A</td>
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<td>---------</td>
</tr>
<tr>
<td>A6-wt</td>
<td>A6-D27A</td>
<td>A6-R28A</td>
</tr>
<tr>
<td>A6-wt</td>
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<td>A6-R28A</td>
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<td>A6-D27A</td>
<td>A6-R28A</td>
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<tr>
<td>A6-wt</td>
<td>A6-D27A</td>
<td>A6-R28A</td>
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<tr>
<td>A6-wt</td>
<td>A6-D27A</td>
<td>A6-R28A</td>
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<td>A6-wt</td>
<td>A6-D27A</td>
<td>A6-R28A</td>
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<td>A6-wt</td>
<td>A6-D27A</td>
<td>A6-R28A</td>
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### 3.3 Templates based on the T1 scTv and variants

<table>
<thead>
<tr>
<th>Template Clone(s)</th>
<th>Type</th>
<th>Date</th>
<th>Purpose</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1-S8</td>
<td>SDM</td>
<td>11 FEB 2010</td>
<td>Introduce Q80R&lt;sub&gt;α&lt;/sub&gt; mutation to determine if stability is conferred</td>
<td><strong>FOR:</strong> 5'- TCA GTA TGT TTC ACT GCT GAT TAG AGA TTC TAG ACC ATC TGA TTC GGC C -3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>REV:</strong> 5'- GGC TGA ATC AGA TGG TCT AGA ATC TCT TAT TAG C -3'</td>
</tr>
<tr>
<td>T1-S18</td>
<td>DL</td>
<td>16 FEB 2010</td>
<td>Generation of the T1-S18 CDR3 library β&lt;sub&gt;1&lt;/sub&gt;</td>
<td><strong>FOR:</strong> 5'- GGA AGA TTC TGG TGT GTA TTT TTT CCG CTC TTC ANN SNN SNN SNN SNN SGT TG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>REV:</strong> 5'- TGA AGA GGA AGA AAA ATA CAC ACC AGA ATC TTC CAG TCG TGC C -3'</td>
</tr>
<tr>
<td></td>
<td>DL</td>
<td>16 FEB 2010</td>
<td>Generation of the T1-S18 CDR3 library β&lt;sub&gt;2&lt;/sub&gt;</td>
<td><strong>FOR:</strong> 5'- GGA AGA TTC TGG TGT GTA TTT TTT CCG CTC TTC ACA TGC GGG TCT GNN SNN SNN SNN SNN SNN SNN SNN SS TGG TCG TCT GCT GAT C -3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>REV:</strong> 5'- TGA AGA GGA GCA AAA ATA CAC ACC AGA ATC TTC CAG TCG TGC C -3'</td>
</tr>
</tbody>
</table>
| T1-S8  | SDM  | 04 MAR 2010 | Introduce S40P<sub>α</sub> mutation to determine if stability is conferred | **FOR**: 5'- CTT TTG GTA TCG CCA GTA TCC TGG TAA ATC ACC GGA A -3'  
**REV**: 5'- TTC CGG TGA TTT ACC AGG ATA CTG GCC GTC AAT CCA AAA G -3' |
|---|---|---|---|---|
| T1-S8  | DL  | 26 MAR 2010 | Generation of the T1-S18 CDR3 library<sup>α1</sup> | **FOR**: 5'- CCA TCT GAT TCA GCC ACT TAT CTG TGC GGC GTG NNS NNS NNS NNS NNS CTG ATG TTT GGC GAT GGT ACC CAG CTG GTT CTG GTT -3'  
**REV**: 5'- CAC CGC GCA CAG ATA AGT GGC TGA ATC AGA TGG TCG AGA ATC TCT AAT CAG CAG TGA AAC ATA CTG AGA -3' |
| T1-S8  | DL  | 26 MAR 2010 | Generation of the T1-S18 CDR3 library<sup>α2</sup> | **FOR**: 5'- CCA TCT GAT TCA GCC ACT TAT CTG TGC GGC GTG AAT GAT NNS NNS NNS NNS NNS TTT GGC GAT GGT ACC CAG CTG GTT GTT -3'  
**REV**: 5'- CAC CGC GCA CAG ATA AGT GGC TGA ATC AGA TGG TCG AGA ATC TCT AAT CAG CAG TGA AAC ATA CTG AGA -3' |
| T1-wt  | SC  | 13 JUL 2010 | Sub-cloning into pET28 | **FOR**: 5'- TAT ACC ATG GGC AGC AGC CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG CCG CGC GGC AGC GAA GCT GGT GTT ACT CAA TTC -3'  
**REV**: 5'- TTT AGA ATT CTT AAA TAT TCG GTT TCA CAA CCA G -3' |
| T1-S18  | SC  | 13 JUL 2010 | Sub-cloning into pET28 | **FOR**: 5'- GCG TCT CTG AAC TGT ACT TAT TCA GAT AGA GGT TCT NNS TCT TTC TTT TGG TAT CGC CA G TAT CCT GGT -3'  
**REV**: 5'- AAG AAA GAT TGA GAA CCT GCA TCT GAA TAA GTA CAG TTC AGA GAC GC -3' |
| T1-S18  | DL  | 10 NOV 2011 | Generation of the T1-S18.45 position Q31<sub>α</sub> library | **FOR**: 5'- GTA CTT ATT CAG ATA GAG GTT CTG CAT CTT TTT GTT ATC GCC AGT -3'  
**REV**: 5'- ACT GCC GAT ACC AAA AGA AAG ATG CAG AAC CTC TAT CTG AAT AAG TAC -3' |
| T1-S18  | DL  | 10 NOV 2011 | Generation of the T1-S18.45 position Y51<sub>α</sub> library | **FOR**: 5'- GCG AAC TGA TCT TGT CTA TCG CTT CAA ACG GGT ATA AGG AAG -3'  
**REV**: 5'- CTT CCT TAT CGC CGT TTG AAG CGA TAG ACA TGA TCA GTT CCG -3' |
| T1-S18  | DL  | 10 NOV 2011 | Generation of the T1-S18.45 position S52<sub>α</sub> library | **FOR**: 5'- CGG AAC TGA TCT TGT CTA TCG CTT CAA ACG GGT ATA AGG AAG -3'  
**REV**: 5'- CCT TAT CGC CGT TTG CAT AGA TAG ACA TGA TCA GTT CCG -3' |
| T1-S18  | DL  | 10 NOV 2011 | Generation of the T1-S18.45 position Y51<sub>α</sub> library | **FOR**: 5'- CTT GGT AAA TCA CCG GAA CTG ATC AGT TCT NNS TCT TTT TGG TAT CGC TAT CCT GGT -3'  
**REV**: 5'- AGA ACC TCT ATC TGA AGT ACA GTT CAG AGA CGC AAT GGC TCC CCG CAC TGA TGG AGA ACC -3' |
### 3.4 Templates based on the WT1-P22 scTv and variants

<table>
<thead>
<tr>
<th>Template Clone(s)</th>
<th>Type</th>
<th>Date</th>
<th>Purpose</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT1-D13</td>
<td>DL</td>
<td>28 JUL 2010</td>
<td>Generation of the WT1 CDR3 library</td>
<td><strong>FOR:</strong> 5'- CAA CCG TCA GAT AGC GCC ACT TGC TGG TGT ATT CAC CGC TCT GGGA TTA ATG TCA GAC GGA TGA GTT CTA GAG AGA AAG GAC ATA AGG AG -3'</td>
</tr>
<tr>
<td>WT1-D15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT1-D13.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-S18.45</td>
<td>5'</td>
<td>25 JAN 2012</td>
<td>Generate amplicon for sequencing with unique barcode (MID)</td>
<td><strong>FOR:</strong> 5'- CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG CGA GGA GCA CCC GCA CAG ATA AGT GAC TG -3'</td>
</tr>
<tr>
<td>Q31</td>
<td>5'</td>
<td>25 JAN 2012</td>
<td>Generate amplicon for sequencing with unique barcode (MID)</td>
<td><strong>FOR:</strong> 5'- CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG CGA GGA GCA CCC GCA CAG ATA AGT GAC TG -3'</td>
</tr>
<tr>
<td>T1-S18.45</td>
<td>5'</td>
<td>25 JAN 2012</td>
<td>Generate amplicon for sequencing with unique barcode (MID)</td>
<td><strong>FOR:</strong> 5'- CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG CGA GGA GCA CCC GCA CAG ATA AGT GAC TG -3'</td>
</tr>
<tr>
<td>Y51</td>
<td>5'</td>
<td>25 JAN 2012</td>
<td>Generate amplicon for sequencing with unique barcode (MID)</td>
<td><strong>FOR:</strong> 5'- CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG CGA GGA GCA CCC GCA CAG ATA AGT GAC TG -3'</td>
</tr>
<tr>
<td>T1-S18.45</td>
<td>5'</td>
<td>25 JAN 2012</td>
<td>Generate amplicon for sequencing with unique barcode (MID)</td>
<td><strong>FOR:</strong> 5'- CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG CGA GGA GCA CCC GCA CAG ATA AGT GAC TG -3'</td>
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<tr>
<td>Y51</td>
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<td>25 JAN 2012</td>
<td>Generate amplicon for sequencing with unique barcode (MID)</td>
<td><strong>FOR:</strong> 5'- CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG CGA GGA GCA CCC GCA CAG ATA AGT GAC TG -3'</td>
</tr>
<tr>
<td>T1-S18.45</td>
<td>5'</td>
<td>25 JAN 2012</td>
<td>Generate amplicon for sequencing with unique barcode (MID)</td>
<td><strong>FOR:</strong> 5'- CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG CGA GGA GCA CCC GCA CAG ATA AGT GAC TG -3'</td>
</tr>
<tr>
<td>SDM</td>
<td>5'</td>
<td>28 JUN 2012</td>
<td>Generate T1-S18.45 D27Aα</td>
<td><strong>FOR:</strong> 5'- GTC TCT GAA TGTA TTA TTC AGC TAG AGG TTC TCA ATC TTT TT -3'</td>
</tr>
<tr>
<td>T1-S18.45</td>
<td>5'</td>
<td>28 JUN 2012</td>
<td>Generate T1-S18.45 D27Yα</td>
<td><strong>FOR:</strong> 5'- CGT CTC TGA ACT GTA CTT ATT CAT ATA GAG GTT CTC AAT CTT TCT TT -3'</td>
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<tr>
<td>SDM</td>
<td>5'</td>
<td>28 JUN 2012</td>
<td>Generate T1-S18.45 D27Wα</td>
<td><strong>FOR:</strong> 5'- CGT CTC TGA ACT GTA CTT ATT CAT ATA GAG GTT CTC AAT CTT TCT TT -3'</td>
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<td>T1-S18.45</td>
<td>5'</td>
<td>22 MAR 2013</td>
<td>Generate T1-S18 Y51Aα in pET28</td>
<td><strong>FOR:</strong> 5'- CGT CTC TGA ACT GTA CTT ATT CAT ATA GAG GTT CTC AAT CTT TCT TT -3'</td>
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<tr>
<td>Code</td>
<td>Type</td>
<td>Date</td>
<td>Description</td>
<td>FOR Forward Primer</td>
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<tr>
<td>WT1-D13</td>
<td>DL</td>
<td>28 JUL 2010</td>
<td>Generation of the WT1 CDR3 library α2</td>
<td>FOR: 5'-CAA CCG TCA GAT AGC GCC ACT TAC CTG TGC GCC GCC GAT AAT AGC GCC GGC GAT TTA ATG -3'</td>
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<td>WT1-D13</td>
<td>DL</td>
<td>28 JUL 2010</td>
<td>Generation of the WT1 CDR3 library β1</td>
<td>FOR: 5'-AGT ACC AAT CAA ACT TCC ATG TAC CTG TGT GCA NNS NNS NNS NNS GAA CAG TTT TTC GGC CCA GGT ACA AGA TTA ACG GTG -3'</td>
</tr>
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<td>WT1-D13</td>
<td>DL</td>
<td>28 JUL 2010</td>
<td>Generation of the WT1 CDR3 library β2</td>
<td>FOR: 5'-AGT ACC AAT CAA ACT TCC ATG TAC CTG TGT GCA AGC AGT TCC NNS NNS NNS NNS NNS NNS TTC GGC CCA GGT ACA AGA TTA ACG GTG -3'</td>
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<td>WT1-P22</td>
<td>DL</td>
<td>01 DEC 2010</td>
<td>Generation of the WT1 CDR1α library</td>
<td>FOR: 5'-ATT GCA TCA TTG AAC TGC ACC TAT AGC GAT CGT NNS NNS NNS NNS TTC TTT TGG TAT AGA CAG TAC AGT GGC AAA TCC CCG -3'</td>
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<td>WT1-D13.1</td>
<td>SDM</td>
<td>15 JUN 2011</td>
<td>Generation of WT1-D13.0.1</td>
<td>FOR: 5'-ATT GAA CTG CAC CTA TAG CGA TCG TGT TTC GCA GTC GTT TTT TTG GTA TAG ACA GTA CAG -3'</td>
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<tr>
<td>WT1-D13.1.1</td>
<td>SC</td>
<td>15 JUN 2011</td>
<td>Sub-cloning into pET28</td>
<td>FOR: 5'-TAT ACC ATG GGC AGC AGC CAT CAT CAT CAT CAT CAC AGC AGC GGC CTC GTG CCG CGC GCC AGC AGT GTT AAA GTG ACA CAA TCT -3'</td>
</tr>
<tr>
<td>WT1-D13.1.1</td>
<td>SDM</td>
<td>07 SEP 2011</td>
<td>Generate WT1-D13.1.1 Y51α library</td>
<td>FOR: 5'-CCC GGA ACT GAT TAT GTC TAT CGC CTC AAA TGG CCA TAA AGA AGA T -3'</td>
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<tr>
<td>WT1-D13.1.1</td>
<td>DL</td>
<td>10 NOV 2011</td>
<td>Generation of the WT1-D13.1.1 CDR1α position 29-31 library</td>
<td>FOR: 5'-GCA TCA TTG AAC TGC ACC TAT AGC GAT CGT NNS NNS NNS TCG TTC TTT TGG TAT AGA CAG TAC AGT GCC -3'</td>
</tr>
<tr>
<td>WT1-D13.1.1</td>
<td>DL</td>
<td>10 NOV 2011</td>
<td>Generation of the WT1-D13.1.1 position Y51α library</td>
<td>FOR: 5'-GGC AAA TCC CCG GAA CTG ATT ATG TCT ATC NNS TCA AAT GGC GAT AAA GAA GAT GGT CGC TTT ACC GCT -3'</td>
</tr>
</tbody>
</table>
| WT1- D13.1.1  
CDR1α position 29-31 library (Pre-sort) | 454 | 25 JAN 2012 | Generate amplicon for 454 sequencing with unique barcode (MID) | FOR: 5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG CGT GTC TCT AGG TCT GTC CGA TGA TGC TAA GAA AGA TGC TGC T -3'  
REV: 5'-CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG ACC CGC GTT ATT CGC GGC GCA CAG GTA AGT GGC -3' |
| WT1- D13.1.1  
CDR1α position 29-31 library (Post-20 nM sort) | 454 | 25 JAN 2012 | Generate amplicon for 454 sequencing with unique barcode (MID) | FOR: 5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG CTC GCG TGT CGG TCT CGA TGA TGC TAA GAA AGA TGC TGC T -3'  
REV: 5'-CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG ACC CGC GTT ATT CGC GGC GCA CAG GTA AGT GGC -3' |
| WT1- D13.1.1  
Y51α library (Pre-sort) | 454 | 25 JAN 2012 | Generate amplicon for 454 sequencing with unique barcode (MID) | FOR: 5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG TAG TAT CAG CGG TCT CGA TGA TGC TAA GAA AGA TGC TGC T -3'  
REV: 5'-CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG ACC CGC GTT ATT CGC GGC GCA CAG GTA AGT GGC -3' |
| WT1- D13.1.1  
Y51α library (Post-sort) | 454 | 25 JAN 2012 | Generate amplicon for 454 sequencing with unique barcode (MID) | FOR: 5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG ATC AGA CAC GAG CGG CCC ATT AAG TGT TCC GGA AGG TGC T -3'  
REV: 5'-CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG ACC CGC GTT ATT CGC GGC GCA CAG GTA AGT GGC -3' |
| WT1- D13.1.1  
SC | 09 OCT 2012 | Sub-cloning into pET28 with BirA tag | FOR: (same as above from 15-JUN-2011)  
REV: 5'-TTT AGA ATT CTT ATT CGT GCC ATT CGA TTT TCT GAG CCT CGA AGA TGT CGT TCA GAC CGC CAC CGA TGT GAG GCT TAA CCA TTA A -3' |

### 3.5 Templates based on other constructs

<table>
<thead>
<tr>
<th>Template Clone(s)</th>
<th>Type</th>
<th>Date</th>
<th>Purpose</th>
<th>Primer Sequences</th>
</tr>
</thead>
</table>
| HLA-A2 α1α2 module | SC | 13 MAY 2010 | To add overhangs for sub-cloning into pCT302 | FOR: 5'-GTA AAA CGA CGG CCA GTG -3'  
REV: 5'-GGA AAC AGC TAT GAC CAT G -3' |
APPENDIX B

REAGENTS AND SOLUTIONS

B.1 Yeast display strain, plasmid, and primers

1. *Saccharomyces cerevisiae* yeast display strain EBY100 (a GAL1-AGA1::URA3 ura3-52 trp1 leu2Δ1 his3Δ0200 pep4::HIS2 prb1Δ1.6R can1 GAL)

2. pCT302 yeast display vector

3. pCT302 standard primers
   a. Splice 4L (forward): 5’ GGCAGCCCCATAAACACACAGTAT
   b. YRS (reverse): Rev 3’ CGAGCTAAAGTACAGTGGG
   c. T7 (reverse): Rev 3’ TAATACGACTCACTATAG

B.2 DNA purification

1. Zymoprep kit II (Zymo Research)

2. QIAprep spin miniprep kit (Qiagen)

3. QIAquick gel extraction kit (Qiagen)

4. Silica bead DNA gel extraction kit (Thermo Scientific)

5. QIAquick PCR purification kit (Qiagen)

6. DNA clean & concentrator-100 kit (Zymo Research)

7. Pellet paint co-precipitant (Novagen)

8. Agencourt AMPure XP PCR purification beads (Beckman Coulter)
B.3 Restriction enzymes and ligation

1. BglII
2. DpnI
3. EcoRI
4. Ncol
5. Nhel
6. Xhol
7. T4 DNA ligase
8. Calf intestinal alkaline phosphatase (CIP)

B.4 PCR

1. FastStart high fidelity PCR system (Roche)
2. dATP solution
3. dCTP solution
4. dGTP solution
5. dTTP solution
6. Deoxynucleotide (dNTP) solution mix (New England Biolabs)
7. PfuTurbo DNA polymerase (Agilent)
8. Taq DNA polymerase (Invitrogen)
9. VentR DNA polymerase (New England Biolabs)

B.5 LiOAc yeast heat shock transformation

1. 50% PEG 3350: dissolve 5 g PEG 3350 to a final volume of 10 mL ddH₂O, sterile filter and store at room temperature for up to 6 months.
2. **1 M LiOAc**: dissolve 16.5 g LiOAc in 250 mL ddH$_2$O, sterile filter and store at room temperature for up to 6 months.

3. **10X TE**: dissolve 121 mg Tris (10 mM) and 29 mg EDTA (1 mM) in 100 mL ddH$_2$O, sterile filter, and store at room temperature for up to 6 months.

4. **Single-stranded carrier**: Dissolve 200 mg salmon sperm DNA (Sigma) in 100 mL 1X TE buffer, aliquot into 1 mL stocks, and store at -20°C.

**B.6 Electrocompetent *E. coli* strains**

1. For DNA amplification: Sub-cloning efficiency DH5α competent cells (Invitrogen)

2. For DNA ligation: Turbo competent *E. coli* (New England Biolabs)

3. For protein expression: BL21(DE3) competent *E. coli* (New England Biolabs)

**B.7 Yeast media**

1. **YPD media**: Dissolve 10 g yeast extract, 20 g bacto-peptone, and 20 g dextrose, bring volume to 1 L with ddH$_2$O, autoclave, and store at room temperature for up to 1 month.

2. **YPD plates**: Dissolve 10 g yeast extract, 20 g bacto-peptone, 15 g agar, and 20 g dextrose, bring volume to 1 L dH$_2$O, and autoclave. Cool to ~55°C and pour ~25 mL into 100 mm X 15 mm plates. Cool and store at +4°C for up to 1 month.

3. **SD-CAA media**: Dissolve 14.8 g sodium citrate, 4.2 g citric acid monohydrate, 5 g casamino acids, 6.7 g yeast nitrogen base (without amino acids), 20 g
dextrose, and 10 mL penicillin-streptomycin (10,000 U/mL), bring volume to 1 L with ddH₂O, sterile filter, and store at 4°C for up to 6 months.

4. SD-CAA plates: Dissolve 91.1 g sorbitol, 7.5 g agar, 7.4 g sodium citrate, and 2.1 g citric acid monohydrate in 400 mL of ddH₂O, autoclave, and cool to ~55°C. In a separate container combine 2.5 g casamino acids, 10 g dextrose and 3.35 g yeast nitrogen base (without amino acids) to 100 mL of ddH₂O, sterile filter, and add to cooled autoclaved solution. Mix and pour ~25 mL into 100 mm X 15 mm plates. Cool and store at +4°C for up to 6 months.

5. SG-CAA media: Dissolve 14.8 g sodium citrate, 4.2 g citric acid monohydrate, 5 g casamino acids, 6.7 g yeast nitrogen base (without amino acids), 20 g galactose, and 10 mL penicillin-streptomycin (10,000 U/mL), bring volume to 1 L with ddH₂O, sterile filter, and store at 4°C for up to 6 months.

**B.8 Yeast library**

1. 1 M sorbitol: dissolve 45.6 g sorbitol in 250 mL ddH₂O, sterile filter, and store at 4°C for up to 6 months.

2. 1 M sorbitol/1 mM CaCl₂: dissolve 45.5 g sorbitol and 27 mg of CaCl₂ in 250 mL ddH₂O, sterile filter, and store at 4°C for up to 6 months.

3. 0.1 M LiAc/10 mM DTT: dissolve 1.65 g lithium acetate (LiAc) and 0.386 g dithiothreitol (DTT) in 250 mL ddH₂O, sterile filter, and cool to 4°C for immediate use.

4. 0.2 cm electroporation cuvettes
B.9 Yeast staining

1. Phosphate-buffered saline (PBS): Dissolve 8 g NaCl, 0.2 g KCl, 1.15 g \( \text{Na}_2\text{HPO}_4\cdot7\text{H}_2\text{O} \), and 0.2 g \( \text{KH}_2\text{PO}_4 \) (anhydrous); bring volume up to 1 L with \( \text{ddH}_2\text{O} \), adjust pH to 7.4, autoclave, and store at room temperature.

2. PBS/1% BSA: Dissolve 10 g bovine serum albumin (BSA) in 1 L PBS, sterile filter, and chill to 4°C.

3. Anti-c-myc, chicken IgY fraction (Invitrogen cat. no. A12181)

4. HA.11 clone 16B12 monoclonal antibody (anti-HA) (Covance cat. no. MMS-101P)

5. AlexaFluor 647 goat anti-chicken IgG (H+L) (Molecular Probes cat. no. A-21449)

6. Streptavidin-phycoerythrin (BD Pharmingen cat. no. 554061)

7. AlexaFluor 647 F(ab')2 fragment of goat anti-mouse IgG (H+L) (Molecular Probes cat. no. A-21237)

8. Goat-anti-mouse IgM APC (Life Technologies cat. no M31505)

9. BD DimerX HLA-A2:Ig recombinant fusion protein, human (BD Pharmingen) cat. no. 551263)

10. Anti-Vα2 antibody (Generated in the Kranz lab)

11. Antibodies against TCR Vβ

   a. Anti-hVβ3 FITC antibody (clone CH92; Beckman-Coulter)

   b. Anti-hVβ3.1 FITC antibody (clone 8F10; Thermo Scientific)

   c. Anti-Vβ14 antibody (clone CAS1.1.3; Beckman-Coulter)
d. Anti-Vβ16 antibody (clone TAMAYA1.2; Beckman-Coulter)
e. Anti-hVβ20 antibody (clone ELL1.4; Beckman-Coulter)

B.10 Expression plasmids

1. HLA-A2 heavy chain (HLA-A2bsp in pHN1); obtained from the from University of Massachusetts Medical School Tetramer Facility
2. HLA-A2 light chain (β2 microglobulin in pHN1); obtained from the NIH Tetramer Facility
3. pET28b expression vector

B.11 Bacterial expression media

1. Luria broth (LB): Dissolve 5 g yeast extract, 10 g tryptone, and 10 g NaCl in 1 L ddH2O, autoclave, and store at room temperature.
2. Ampicillin (100 mg/mL): Stock solution can be made by dissolving 1 g into 10 mL ddH2O, sterile filtering, and freezing individual aliquots at -20°C.
3. Kanamycin (50 mg/mL): Stock solution can be made by dissolving 0.5 g into 10 mL ddH2O, sterile filtering, and freezing individual aliquots at -20°C.
4. LB + ampicillin (100 µg/mL) plates: Dissolve 5 g yeast extract, 10 g tryptone, 10 g NaCl, and 15 g agar in 1 L dH2O and autoclave. Cool to ~55°C, then add 1 mL ampicillin stock at 100 mg/mL, swirl, then pour ~25 mL into 100 mm X 15 mm plates. Cool and store at +4°C.
5. LB + kanamycin (50 µg/mL) plates: Dissolve 5 g yeast extract, 10 g tryptone, 10 g NaCl, and 15 g agar in 1 L dH2O and autoclave. Cool to ~55°C, then
add 1 mL kanamycin stock at 50 mg/mL, swirl, then pour ~25 mL into 100 mm X 15 mm plates. Cool and store at +4°C.

B.12 Inclusion body isolation

1. Lysis buffer: 50 mM Tris base, 100 mM NaCl, 0.1% NaN₃, 1% Triton X-100, 10 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF, dissolved in 1 mL isopropanol), bring volume to 1 L with ddH₂O and adjust pH to 8.0 with hydrochloric acid. Buffer can be stored without DTT and PMSF for up to 6 months.

2. Osmotic shock buffer: 20 mM Tris base and 2.5 mM ethylenediaminetetraacetic acid (EDTA); bring volume up to 1 L with ddH₂O, adjust pH to 8.0, and chill to 4°C.

3. Osmotic shock buffer with triton: Add 0.5% Triton X-100 to 1 L osmotic shock buffer and chill to 4°C.

4. Urea extraction buffer: 8 M urea, 25 mM MES, 10 mM EDTA, and 0.1 mM dithiothreitol (DTT) in 10 mL at pH 6.0.

5. Guanidine extraction buffer: 8 M guanidine-HCl, 50 mM Tris, 5 mM EDTA, 5 mM dithiothreitol (DTT) in 10 mL at pH 8.0.

B.13 scTCR refold

1. scTCR refold buffer: 3 M urea, 50 mM Tris base; bring volume up to 400 mL with ddH₂O, adjust pH to 8.0 with HCl, and chill to 4°C.

2. scTCR dilution buffer: 200 mM NaCl, 50 mM Tris base; bring volume up to 3 L with ddH₂O, adjust pH to 8.0 with HCl, and chill to 4°C.
3. Oxidized glutathione
4. Reduced glutathione
5. Phenylmethylsulfonyl fluoride (PMSF)
6. Ni-NTA agarose (Qiagen)
7. Elution buffer: 500 mM imidazole, 2.5 mM EDTA solution in 1 X PBS pH to 8.0.

B.14 MHC refold

1. MHC refold buffer: 400 mM Tris, 400 mM L-Arg, 2 mM EDTA; bring volume up to 200 mL with ddH$_2$O, adjust pH to 8.0, and chill to 4°C.
2. Injection buffer: 3 M guanidine-HCl, 10 mM sodium acetate, and 10 mM EDTA; bring volume up to 250 mL with ddH$_2$O and adjust pH to 4.2. Aliquots can be frozen and stored at -20°C.
3. Dialysis buffer: 20 mM Tris in 3 L ddH$_2$O, adjust pH to 8.0, and chill to 4°C.
4. Regenerated cellulose 6-8 MWCO dialysis tubing
5. Amicon Ultra-4 centrifugal filter units with Ultrace1-10 membrane (Amicon)

B.15 TCR and MHC biotinylation, purification, and quantification

1. *In vitro* biotinylation kit (Avidity)
2. TCR HPLC buffer: 1 X PBS, adjust to pH 8.0, and degas.
3. MHC HPLC buffer: 20 mM tris and 50 mM NaCl in 1 L ddH$_2$O, adjust pH to 8.0, and degas.
4. EDTA stock: 0.5 M EDTA in 50 mL ddH$_2$O, adjust pH to 8.0, and sterile filter.
5. BCA protein assay kit (Pierce)
B.16 MHC-restricted peptides

1. UV-cleavable peptide, KILGFVFJV, where J is the photolabile amino acid residue, prepared by standard Fmoc-peptide solid phase synthesis using commercially available Fmoc-3-amino-3-(2-nitro)phenyl propionic acid as a building block; store in the dark at -20 °C.

2. HLA-A2-restricted peptide(s) (20 mg/mL): Dissolve 20 mg of lyophilized peptide into 1 mL DMSO; store at -20°C.
   a. gp100_{209–217}: ITDQVPFSV
   b. HBV-Env_{183–191}: FLLTRILTI
   c. MART-1_{27-35} (MART-1_9mer): AAGIGILTV
   d. MART-1_{27-35} A_{28L}: ALGIGILTV
   e. MART-1_{26–35} A_{27L} (MART-1_10mer): ELAGIGILTV
   f. MDM2_{53-60}: VLFYLGQY
   g. NYESO_{157–165}: SLLMWITQC
   h. NYESO_{157–165} Q_{8NC9V} (NYESO-Val): SLLMWITNV
   i. Preproinsulin_{15-24} (PPI): ALWGPDPAAA
   j. SL9_{77-85} (HIV-gag): SLYNTVATL
   k. Survivin_{95–104}: LTLGEFLKL
   l. Survivin_{95-104} T_{2M}: LMLGEFLKL
   m. Tax_{11–19}: LLFGYPVYV
   n. Tax_{11–19} F_{3A}: LLAGYPVYV
   o. Tax_{11–19} F_{3G}: LLGGYPVYV
p. Tax\textsubscript{11–19} \textsuperscript{Y5A}: LLFGAPVVV

q. Tax\textsubscript{11–19} \textsuperscript{Y8A}: LLFGYPVAV

r. Tax\textsubscript{11–19} \textsuperscript{Y8T}: LLFGYPVT

s. Tax\textsubscript{11–19} F3\textsuperscript{AY8T}: LLAGYPVT

t. Tax\textsubscript{11–19} F3\textsuperscript{AY5AY8T}: LLAGAPVT

u. Tel1\textsubscript{p549–557}: MLWGYLQYV

v. WT\textsubscript{126–134}: RMFNPAPYL

3. HLA-A2-restricted peptide(s) (2 mg/mL): Perform a 1:10 dilution of 20 mg/mL peptide stock in DMSO in PBS; store at -20°C.

**B.17 Magnetic selection**

1. PBSM buffer: 8 g NaCl, 0.2 g KCl, 1.44 g Na\textsubscript{2}PO\textsubscript{4}, 0.24 g KH\textsubscript{2}PO\textsubscript{4}, 5 g bovine serum albumin, and 744 mg EDTA in 1 L dH\textsubscript{2}O, adjust to pH 7.4, sterile filter and store at 4°C.

2. Microbeads conjugated to anti-mouse IgG (Miltenyi Biotec cat. no. 130-048-401)

3. Microbeads conjugated to strepavidin (Miltenyi Biotec cat. no. 130-048-101)

4. Microbeads conjugated to anti-biotin (Miltenyi Biotec cat. no. 130-090-485)

5. LS Columns (Miltenyi Biotec cat. no. 130-042-401)

**B.18 DNA quantification, analysis, site-directed Mutagenesis, and 454 sequencing**

1. Qubit dsDNA HS assay kit (Life Technologies)

2. Agilent DNA 7500 kit (Agilent Technologies)
3. QuikChange II or QuikChange lightening site-directed mutagenesis kit (Agilent)

4. GS FLX titanium sequencing kit XL+ (Roche)

5. GS FLX titanium PicoTiter plate kit 70x75 (Roche)

B.19 Equipment

1. Thermocycler

2. Two temperature controlled incubator shakers

3. Electroporator (BioRad Gene Pulser II electroporation system)

4. Flow cytometer and FACS apparatus

5. Amicon 8400 stirred ultrafiltration cell (Millipore) with regenerated cellulose ultrafiltration discs, YM-10, 10 kDa NMWL, 76mm (Millipore cat. no. 13642)

6. Superdex 200 10/300 GL gel filtration column (GE Biosciences cat. no. 17517501)

7. CL-1000 ultraviolet crosslinker (UVP, LLC)

8. MidiMACS separator (Miltenyi Biotec)

9. MACS multiStand (Miltenyi Biotec)

10. Qubit 2.0 fluorimeter (Life Technologies)

11. Agilent 2100 bioanalyzer (Agilent Technologies)

12. Roche/454 genome sequencer FLX+ (Roche)