Structure-Guided T Cell Receptor Mutations that Alter Antigen Specificity, Cross-Reactivity, and Polyfunctional Phenotypes in Gene-Modified T Cells

Kendra Foley

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STRUCTURE-GUIDED T CELL RECEPTOR MUTATIONS THAT ALTER ANTIGEN SPECIFICITY, CROSS-REACTIVITY, AND POLYFUNCTIONAL PHENOTYPES IN GENE-MODIFIED T CELLS

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PROGRAM IN INTEGRATIVE CELL BIOLOGY

BY
KENDRA C. FOLEY
CHICAGO, ILLINOIS
AUGUST 2019
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<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>ACK</td>
<td>Ammonium-Chloride-Potassium</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AF</td>
<td>Alexa Fluor</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>APC</td>
<td>Allophycocyanin</td>
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<tr>
<td>AIRE</td>
<td>Autoimmune regulator</td>
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<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guerin</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>BV</td>
<td>Brilliant Violet</td>
</tr>
<tr>
<td>C</td>
<td>Constant</td>
</tr>
<tr>
<td>CAR</td>
<td>Chimeric antigen receptor</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity-determining region</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CLIP</td>
<td>Class II associated invariant chain peptide</td>
</tr>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte-associated protein 4</td>
</tr>
<tr>
<td>D</td>
<td>Diversity</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMF5</td>
<td>T cell receptor recognizing the melanoma antigen, MART-1</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative</td>
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<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DP</td>
<td>Double Positive</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FcR</td>
<td>Fc receptor</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>FRET</td>
<td>Fluorescent resonance energy transfer</td>
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<tr>
<td>FSC</td>
<td>Forward scatter</td>
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<td>GaLV</td>
<td>Gibbon ape leukemia virus</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>gp100</td>
<td>Premelanosome protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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</table>
GVHD  Graft versus host disease
H  Heavy
HCV  Hepatitis C virus
HCV 1406  T cell receptor recognizing the Hepatitis C virus protein NS3 1406-1415 amino acid sequence
HLA  Human leukocyte antigen
HPLC  high-performance liquid chromatography
HREEP 5  Human receptor expression enhancing protein 5
HRP  Horseradish peroxide
HSV  Herpes simplex virus
IFN-α  Interferon-α
IFN-γ  Interferon-γ
Ig  Immunoglobulin
IL-2  Interleukin-2
IL-4  Interleukin-2
IL-5  Interleukin-6
IL-6  Interleukin-6
IL-9  Interleukin-9
IL-12  Interleukin-12
IL-13  Interleukin-13
IL-15  Interleukin-15
IL-17  Interleukin-17
IL-22  Interleukin-2
IL-17A  Interleukin-17A
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<td>IL-22</td>
<td>Interleukin-22</td>
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<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
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<tr>
<td>IU</td>
<td>International Unit</td>
</tr>
<tr>
<td>J</td>
<td>Joining</td>
</tr>
<tr>
<td>L</td>
<td>Light</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LAG-3</td>
<td>Lymphocyte-activation gene 3</td>
</tr>
<tr>
<td>LAK</td>
<td>Lymphocyte-activated killer</td>
</tr>
<tr>
<td>Lck</td>
<td>Lymphocyte-specific protein tyrosine kinase</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>lmrA</td>
<td>Lincomycin resistance protein</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeats</td>
</tr>
<tr>
<td>M tub.</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
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<td>MART-1</td>
<td>Melanoma antigen recognized by T cells 1</td>
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<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
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<td>MEL</td>
<td>Melanoma</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>Murinized</td>
<td>Of mouse origin</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
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xvi
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<td>NHEJ</td>
<td>Nonhomologous end joining</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKG2D</td>
<td>Natural killer group 2 member D</td>
</tr>
<tr>
<td>NS3</td>
<td>Nonstructural protein 3</td>
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<tr>
<td>NSG</td>
<td>Nod scid gamma</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocyte</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBSA</td>
<td>Phosphate-buffered saline with bovine serum albumin</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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<tr>
<td>PD-1</td>
<td>Programmed cell death 1</td>
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<td>PDL-1</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll protein</td>
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<tr>
<td>pM</td>
<td>Picomolar</td>
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<tr>
<td>PMA</td>
<td>Phorbol 12-Myristate 13-Acetate</td>
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<tr>
<td>PMEL</td>
<td>Premelanosome protein</td>
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<tr>
<td>pMHC</td>
<td>Peptide-major histocompatibility complex</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
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<tr>
<td>REP</td>
<td>Rapid expansion protocol</td>
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<td>rhIL-2</td>
<td>Recombinant human IL-2</td>
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<td>-------------</td>
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</tr>
<tr>
<td>RS</td>
<td>Recombination signal</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
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<td>SA</td>
<td>Splice acceptor</td>
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<td>SHP-1</td>
<td>Src homology 2 domain-containing phosphatase 1</td>
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<tr>
<td>SPICE</td>
<td>Simplified Presentation of Incredibly Complex Events</td>
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<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
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<td>SSC</td>
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<td>STAT1</td>
<td>Signal transducer and activator of transcription 1</td>
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<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
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<td>TIL</td>
<td>Tumor infiltrating lymphocyte</td>
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<td>TIM-3</td>
<td>T cell immunoglobulin, mucin domain 3</td>
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<tr>
<td>TLS</td>
<td>Translation/libration/screw</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
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<tr>
<td>V</td>
<td>Variable</td>
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<td>vs.</td>
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</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
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<tr>
<td>WT</td>
<td>Wildtype</td>
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<tr>
<td>ZAP70</td>
<td>Zeta-chain-associated protein kinase 70</td>
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</table>
ABSTRACT

Adoptive cell transfer of T cell receptor (TCR) gene-modified T cells targeting specific tumor antigens is currently in clinical trials for patients with advanced malignancies. Despite the clinical responses, there are still hurdles to be overcome in achieving an effective and safe therapy. One of the limitations in the success of this type of therapy is the potential for cross-reactivity and unanticipated off-target reactivity which could lead to autoimmunity. Adverse events encompassing these “off-target, off-tumor” cross-reactivities leading to autoimmunity have been seen in patients in different clinical trials. Here, we demonstrate a novel approach to improve antigen specific reactivity and to reduce off-target cross-reactivity by modifying the TCR using structure-guided mutations. This strategy combines mutations that alter the TCR to enhance antigen specificity with mutations that alter the TCR/MHC contact residues to reduce TCR binding with the MHC. Using the DMF5 TCR that targets the melanoma associated antigen MART-1, we examined HLA-A2 restricted cross-reactivity by measuring the polyfunctional T cell response by T cells transduced with strategically mutated DMF5 TCRs against a panel of MART-1 homologs. We further investigated how structure-guided mutations in the DMF5 TCR affected tumor lysis, processed antigen recognition, and 3D vs. 2D affinity. We demonstrate that while modified DMF5 TCRs can enhance on-target specificity, they can also lead to unexpected off-target cross-reactivity. Our data fully supports the notion that affinity is not always an accurate predictor of T cell function and cross reactivity. Moreover, we highlight the importance of rigorous pre-
clinical testing to examine the potential for new specificities and reactivities of modified TCRs. By determining how various TCR mutations can alter functional T cell phenotypes and on-target responses, we can gain a better understanding of the biology of the TCR/pMHC (peptide-MHC) interface and thus how to maximize the efficacy and safety of TCRs to be used in gene-modified T cell in adoptive cell transfer.
CHAPTER ONE
REVIEW OF LITERATURE

Introduction

T cells play a vital role in adaptive immunity in fighting microbial and viral infections as well as malignancies. The initiation and the specificity of T cell immunity lies within the T cell receptor (TCR). Consequently, understanding the different mechanisms associated with TCR antigen recognition, T cell activation, and T cell function that are involved in the adaptive immune response is critical for the success of treatments or therapies that utilize T cells.

Over recent years, immunotherapy has become increasingly prevalent and promising as an instrument to utilize the power of the immune system to fight cancer and disease. Even though the adaptive immune response is capable of recognizing infections and malignancies, the response is not always efficient enough for clearance. Some of the advances in cancer immunotherapy are the result of a variety of different strategies focused on enhancing the immune response via tumor infiltrating lymphocytes (TILs), gene-modified T cells, cytokines, chemokines, mono-clonal antibodies (mAb), and vaccines [1]. Adoptive T cell transfer therapy has been shown to have clinical success in treating melanoma and other malignancies [1]. This process commonly involves isolating TILs, ex vivo expansion, and subsequent administration back into the patient [2]. Albeit, the isolation and further expansion of these TILs can be
difficult and is not always possible in a wide variety of cancers [3]. One alternative strategy that has been clinically successful is the use of genetically modified T cells for adoptive cell transfer. The transduction of T cells or NK (natural killer) cells with chimeric antigen receptors (CARs) or TCRs has demonstrated the ability to redirect the specificity of these cells to recognize tumor and/or viral associated antigens of choice [4]. It is possible to isolate tumor reactive T cell clones from TIL or peripheral blood lymphocyte (PBL) derived T cells and further identify and clone the TCR genes that mediate recognition of tumor associated antigens or viral antigens. In this dissertation we will exclusively study TCR biology by using a retroviral vector encoding TCR genes to redirect the specificity of T cells to recognize a specific tumor antigen. TCR gene-modified T cells for adoptive T cell transfer is a form of a more personalized medicine, since its procedure is unique for each individual patient. As personalized medicine is becoming a more attractive treatment strategy and the methodologies used in this therapy can be applied to a variety of cancers and viral infections, it is imperative to concentrate on making this therapy as clinically efficient and safe as possible.

One of the challenges in using TCR gene-modified T cells that target self-antigens for therapy, is that the TCRs generally harbor a lower affinity. This is a result of negative selection in the thymus during development. Negative selection is critical in order to eliminate potentially autoreactive T cells. Strategies to enhance TCR affinity commonly include yeast and phage display. Affinity enhancement through random TCR mutation can allow for potential unpredicted cross-reactivity. Adverse events, even patient deaths, have been reported where affinity enhanced TCRs were used [5, 6]. Therefore, there is a rising concern in the field in regards to the safety associated with
using TCRs that target self-antigens, and this concern is even further amplified when TCR modifications come in to play. In this dissertation, we have developed a novel strategy to modify the TCR in order to enhance antigen specificity while simultaneously reducing potential cross-reactivity. The structural explanation and further implications on T cell function and therapeutic efficacy of TCR gene-modified T cells will be discussed in this dissertation.

**TCR Diversity**

This dissertation focuses on TCR gene-modified T cells, therefore, it is important to understand T cell development, T cell biology, and T cell genetics. The initiation and the specificity of T cell immunity lies within the TCR. TCR diversity is imperative for responses against a large number and a diverse group of antigens. Consequently, TCR diversity allows for an expansive immune response. TCRs are heterodimers expressed on the T cell surface as either αβ or γδ heterodimers. The extensive repertoire of TCRs is a result of somatic gene rearrangement of the TCR gene loci to allow for recognition of roughly $10^{18}$ epitopes [7]. The TCR gene segments are comprised of the following regions: variable (V), diversity (D), joining (J), and constant (C). Only the TCR beta and delta chains contains the D region [8]. V(D)J recombination occurs in the thymus and is vital for the generation TCRs with diverse recognition. The process of recombination is site-specific and occurs between TCR gene segments that are flanked by recombination signal (RS) sequences. Recombination activating genes-1 and -2 (RAG-1 and RAG-2) bind to the RS sequences and make single strand nicks in the DNA to initiate recombination. First, D-J gene rearrangements occur in the TCR beta or delta chain, followed by V-DJ gene rearrangements [9]. The TCR beta chain will assemble with the
pre-T alpha chain to form a pre-TCR. The TCR alpha chain is subsequently rearranged. Specifically, V-J gene rearrangements occur and are mediated by RAG-1 and RAG-2 [10]. During recombination, diversity is also enhanced at junctional regions via the incorporation of “P” nucleotides and “N” nucleotides and deletion of nucleotides [11-13]. Nonhomologous DNA end-joining (NHEJ) proteins function to then join the rearranged gene fragments [9, 14]. In summary, this recombination is essential for the generation of a large and diverse TCR repertoire in order to allow for the recognition of many pathogens.

**T cell Development**

T cells arise from hematopoietic stems cell progenitors derived from the bone marrow that home to the thymus. Commitment to the T cell lineage occurs upon Notch signaling [15]. This is critical, as mice generated with neonatally induced loss of Notch1 exhibited austere deficiency in thymocyte development [16]. For these studies we focus only on the αβ TCR and thus, we will further describe T cell development and T cell function in regards to the αβ TCR. T cells begin as TCR⁺, CD4⁺, and CD8⁻ and are therefore termed double negative (DN). CD4 and CD8 are co-receptors are found on the surface of T cells and they play an important role in T cell activation. CD4 and CD8 will be described in more detail in a later section. There are four differentiation stages which are classified by various markers on the T cell surface. In the stages DN2 through DN4, the developing T cell expresses a pre-TCR [17]. This pre-TCR consists of a fully rearranged TCR beta chain and a non-rearranged pre-TCR alpha chain in association with the CD3 signaling complex. The pre-TCR induces T cell survival, expansion, and differentiation. The pre-TCR also functions to control allelic exclusion of the TCR beta
locus as well as permitting TCR alpha chain rearrangement [18, 19]. With a fully rearranged αβ TCR on the T cell surface, the T cell becomes double positive (DP), expressing both CD4 and CD8 co-receptors, and will move onto the next step in T cell development [17].

The next step in T cell development consists of positive and negative selection of the T cells, which occurs in the thymic cortex and medullar, respectively. The TCR expressed on a T cell recognizes peptide-MHC complexes on stromal or dendritic cells. If there is no, or weak, recognition by the TCR of MHC and self-antigen, death by neglect will occur. If the TCR binds too strongly to the MHC and self-antigen, the T cell is negatively selected and apoptosis occurs [17]. High affinity TCRs can result in autoreactive T cells and autoimmunity. Thus, negative selection serves to avoid potential autoimmunity and to establish central tolerance. The TCR must recognize self-antigen in the context of MHC via a weak interaction to be positively selected [17]. However, it is critical that non-thymus related antigens are presented in the thymus to prevent autoimmunity in other tissues. The transcription factor encoded by the autoimmune regulator (AIRE) gene has been identified and is critical for the expression of other tissue-restricted antigens [20-23]. Mutations in the human AIRE gene can result in multiple different autoimmune diseases [24-26]. Therefore, AIRE is important for promoting negative selection of T cells with TCR specific for peripheral antigens.

Following positive and negative selection, T cells migrate to the medulla and become single positive, expressing either the CD4 or CD8 co-receptor, depending upon the initial TCR signal [27]. Upon selection, these T cells will migrate into the periphery to elicit an immune response. How TCRs recognize numerous antigens, both self and non-
self, is discussed in a later section. First, it is important to understand how a TCR recognizes its ligand to initiate T cell activation.

**MHC Restriction**

In 1974, Peter Doherty and Rolf Zinkernagel demonstrated the concept of MHC restriction where T cell activation required two concurrent factors, foreign antigen in the context of self-MHC [28]. It was later determined through the crystallization of HLA-A2 by Bjorkman and colleagues, that the MHC presented peptides [29, 30]. This has since been well appreciated as a hallmark in T cell-mediated immunity, as well a unique receptor-ligand interaction. Accordingly, two models have been proposed regarding the drivers of the MHC restriction of TCRs.

The germline-encoded model suggests the TCR genes have co-evolved with the MHC and thus, are selected for inherent motifs important for recognition of the MHC molecules [31]. This model theorizes that multiple “interaction codons” exist and are evolutionarily conserved in the TCR V region and MHC molecules to drive their interaction [32]. The second model, the selection model, suggests that positive selection during development in the thymus imposes MHC reactivity, not intrinsic reactivity to MHC [33]. Specifically, this model suggests MHC restriction is driven by CD4 or CD8 co-receptor binding to the MHC for initiation of TCR signaling through localization of Lck [34, 35]. Structural and functional studies have provided evidence for both of these models and do not exclude the possibility for either.

There are a few pieces of evidence that support the notion that TCRs have evolved to intrinsically recognize MHC molecules. First, TCR complementarity-determining regions (CDRs) are more evolutionarily conserved among species than
immunoglobulin [36]. CDRs are regions of the TCR, specifically in the variable region, that bind pMHC. Studies examining TCR/pMHC crystal structures have revealed evolutionarily conserved residues in the TCR V genes that contact MHC. Results concluded that TCRs frequently utilize amino acids at positions 28, 29, and 31 of the CDR1α chain, positions 50, 51, and 52 of the CDR2α chain, positions 28 and 29 of the CDR1β chain, and position 48 of the CDR2β chain to bind the MHC molecule. For example, position 31 in the TCR CDR1α chain frequently binds at position 155 in the α2 helix of MHC class I or position 70 in the β helix of MHC class II [37]. Also, around 40% of human and mouse TCRs have a tyrosine or phenylalanine at position 31 in the TCR alpha chain [37]. The most conserved residue in published TCR structures is a serine at position 51 in the CDR2α chain. This residue makes contacts with the conserved residues at positions 158 and 151 in the MHC class I α helix [38, 39]. One last example in the TCR alpha chain is position 50 (a tyrosine in 16% of sequenced TCRs) in the CDR2 region. Residues at this TCR position contact position 158 of MHC class I or position 73 of the β helix in MHC class II [37]. In the TCR beta chain, a tyrosine or asparagine at position 29 in the CDR1β often contacts the MHC class I α1 helix [37]. Most notable in the TCR beta chain is a conserved tyrosine at position 48 in the CDR2β that contacts the MHC class I α1 helix. This residue appears to be critical for anchoring the TCR to the MHC [37, 40]. There are numerous other specific examples of conserved residues in TCRs that frequently contact residues in the MHC. Lastly, up to 30% of TCRs display intrinsic reactivity for MHC before completing positive and negative selection in the thymus [41]. In summary, numerous examples of conserved
interactions between TCRs and the MHC molecule suggests that TCRs have evolved to intrinsically recognize MHC molecules.

Another concept that supports the germ-line encoded model is the fact that TCRs generally bind pMHC in the same angled orientation across the MHC. Specifically, the TCR alpha chain lies over the α2 helix or β helix in MHC class I or II, respectively, and the TCR beta chain lies over the α1 or α helix of MHC class I or II, respectively [41]. Furthermore, the pivot point of the TCR centered over the peptide on the MHC is generally the same. In MHC class I, this is positions 4 through 6 of the peptide versus position 5 of the peptide in MHC class II [37]. If recognition of MHC was due to positive selection (hypothesis of the selection model) you might question why the orientation is not commonly reversed. It has been proposed that conserved residues in the CDR1 and CDR2 regions of the TCR are responsible for determining the angle and orientation of the TCR on pMHC, because these conserved interactions occur diagonally and opposite of each other on the helices [37]. In summary, there are numerous features of the TCR/pMHC interaction that suggest TCRs have evolved to intrinsically recognize MHC molecules.

Conversely, there is evidence that also suggests TCRs have not co-evolved to recognize the MHC molecules. First, TCR genes and MHC genes are found on different chromosomes, and no mechanism appears to control co-expression of the proteins [37]. Furthermore, studies have demonstrated cases of antigen recognition in a MHC independent manner [42, 43]. In mice, the deletion of MHC class I, MHC class II, CD4, and CD8 resulted in the expression of TCRs with the ability to recognize epitopes in an MHC independent manner [33]. Lastly, examples of reversed docking of the TCR over
the MHC has been reported [44, 45]. Collectively, studies that analyzed the TCR/pMHC crystal structure and the pre-selection TCR repertoire have resulted in conclusions that provided evidence to support both the germline-encoded model and the selection model for MHC restriction by TCRs. While the concept of MHC restriction is fundamental in immunology, it is also important to understand how antigen is associated with the MHC molecule in a process called antigen presentation.

**Antigen Presentation**

Activation of a T cell requires the engagement of the TCR with peptide in the context of either MHC class I or MHC class II. MHC class I is expressed on all nucleated cells, while MHC class II expression is limited to antigen presenting cells (APCs) such as dendritic cells, macrophages, or B cells. The MHC class I α chain, MHC class II α chain, and MHC class II β chains are each encoded by three genes in humans. The classical MHC genes include HLA-A, -B, and -C for MHC class I and HLA-DR, -DQ, and -DP for MHC class II [46]. MHC is inherited as a haplotype from each parent. Thus, two siblings have a 50% chance of sharing one haplotype, and a 25% chance of being genotypically identical [47]. An exception to this is the possibility of recombination [48]. The two inherited MHC alleles are co-dominantly expressed [49]. MHC genes are extremely polymorphic, with humans having over 800 MHC class I and over 600 MHC class II alleles [50]. MHC genetic variation is proposed to be important for fighting pathogens and overall survival and fitness [51-53]. This polymorphism allows for genetic variation within the MHC region that binds antigen, known as the peptide binding groove, and thus, affects the potential peptide presentation [54, 55].
summary, the extremely polymorphic and co-dominantly expressed MHC genes, are critical for the activation of a T cell-mediated immune response.

Peptides presented in the context of the MHC class I molecule and the MHC class II molecule are derived from two distinct proteolytic mechanisms [46]. For MHC class I peptide binding, proteins in the cytoplasm undergo degradation by the proteasome and are transported to the endoplasmic reticulum (ER) by the transporter associated with antigen processing protein (TAP). Peptides in the ER are loaded onto the MHC class I molecule in the peptide loading complex and then the complex is transported through the Golgi to the cell surface [46, 56]. Alternatively, peptides presented on MHC class II are generally exogenous antigens from outside the cell that are endocytosed. These exogenous antigens are delivered to late endosomes and are processed by cathepsins. At this point, the MHC class II protein and the chaperone HLA-DM protein have passed through the Golgi and into the late endosome [46]. The class II-associated invariant chain peptide (CLIP) plays an important role in stabilizing the peptide free complex, but must be removed to allow peptide loading [57, 58]. Peptides are loaded onto the MHC by HLA-DM and subsequently transported to the cell surface [46]. In summary, antigen presentation occurs via two distinct pathways for MHC class I and MHC class II.

In these studies we focus on TCRs that recognize peptides in the context of MHC class I. Therefore, for the remainder of this dissertation, we will focus exclusively on peptides presented in the context of the MHC class I molecule. MHC class I molecules are comprised of an alpha chain (three extracellular domains - α1, α2, and α3) that is non-covalently associated with β2 microglobulin. The alpha chain is commonly referred
to as the heavy chain and the $\beta_2$ microglobulin is commonly referred to as the light chain. The $\beta_2$ microglobulin lies next to the $\alpha 3$ domain and under the $\alpha 1$ domain [59]. The peptide binding groove is formed by the $\alpha 1$ and $\alpha 2$ domains. These regions are highly polymorphic to allow for the allele specific peptide presentation [60, 61]. The membrane-proximal $\alpha 3$ domain is the binding site for the CD8 co-receptor (conserved among all MHC class I molecules) [62, 63]. MHC class I generally presents peptides of eight to ten amino acids in length [64]. The interactions between the peptide and the MHC are dependent upon charge distribution, geometry, and hydrophobicity of the peptide binding groove [65]. In the heavy chain, six pockets (A-F pockets) in the MHC peptide binding groove exist to accommodate peptide side chains. Specifically, the B-pocket accommodates the N-terminal anchor of the peptide and the F-pocket accommodates the C-terminal anchor of the peptide [65-67]. Most anchor residues are generally considered to be hydrophobic residues, but this can vary depending upon the MHC class I allele. It is possible that antigen presentation favors more hydrophobic regions of proteins, as a correlation has been observed between the immunogenicity and the hydrophobicity of peptides [68]. Previous studies suggested that the exposed hydrophobic domains in proteins significantly enhanced the rate of proteasomal degradation and MHC presentation [69]. This correlation between immunogenicity and hydrophobicity could be of importance when addressing the cross-reactivity of TCRs, a major topic in this dissertation, and will be discussed in future sections.

pMHC Recognition by the TCR

The TCR/pMHC interaction is like any other protein-protein interaction in that it is mainly governed by hydrogen bonds and charges, or van der Waals interactions [70].
The individual van der Waals interactions are often weak, but in combination across the whole TCR/pMHC interface can equate to considerable binding energies. The specificity of the TCR/pMHC interaction is dependent upon, and can be enhanced by hydrogen bonds and salt bridges [71]. On a more macro level, these interactions between the TCR/pMHC are reliant on the CDRs of the TCR. As mentioned previously, CDRs are regions of the TCR, specifically in the variable region, that bind pMHC. In summary, the TCR/pMHC interaction consists of numerous van der Waals interactions and the TCR CDR loops are critical for recognition of the pMHC.

The diversity of the TCR repertoire is critical for an inclusive T cell-mediated immune response. The diversity found in the TCR repertoire is due to the six CDR loops in the αβ TCR. CDR1, CDR2, and CDR3 loops are present in both the TCR alpha chain and the TCR beta chain. The CDR1 and CDR2 loops are germline-encoded versus the hypervariable CDR3 loop [8]. It has been estimated that 75-80% of the TCR/pMHC interaction occurs between the CDR1 and CDR2 regions of the TCR and the MHC helices [71]. As mentioned previously, TCRs generally bind the pMHC class I complex in the same angled orientation across the MHC α helices [37]. The Vα domain generally lies over the amino-end of the peptide and the α2 helix of MHC class I, whereas the Vβ domain generally lies over the carboxyl-end of the peptide and the α1 helix of MHC class I. More specifically, the most variable region of the TCR, the CDR3 region, is positioned in the center of the binding interface in order to make contact with the peptide. It was previously mentioned that antigen presentation favors hydrophobic regions of proteins. Furthermore, peptides with a hydrophobic core are favorable for TCRs to recognize because there is less precise geometry for the CDR loops to match
or engage [72]. The more conserved regions of the TCR, the CDR1 and CDR2 regions, are positioned over the tops of the MHC helices. However, it is still entirely possible for the CDR3 loops to contact the MHC and the CDR1 and CDR2 loops to contact the peptide [71, 73]. The TCR CDR loops are capable of conformational shifts to accommodate the pMHC via their intrinsic flexibility. This has been demonstrated by different TCR conformations in free and bound states, and with the same TCR binding different ligands [74-79]. In summary, the CDR loops of the TCR orient over the pMHC in a conserved manner and dictate any potential binding with the pMHC. TCR binding with the pMHC is the first step for inducing subsequent T cell activation.

**T cell Activation**

T cell activation occurs when a TCR engages with a peptide in the context of the MHC molecule. This TCR/pMHC engagement causes a conformational change in the CD3 signaling complex [80, 81]. The CD3 signaling complex consists of ε, γ, δ, and ζ subunits. These subunits form a CD3εγ heterodimer, a CD3εδ heterodimer, and a CD3ζζ homodimer [82]. Upon antigen recognition via the TCR, the Src-family kinase, Lck, is recruited to the CD3 complex and phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMS) of the CD3ζ chain [83]. The related Src kinase Fyn also can phosphorylate the CD3 ITAMs [84-88]. Zap-70 is then recruited to the phosphorylated ITAMS, is phosphorylated by Lck or itself, and propagates a cascade of downstream signaling pathways [81, 89, 90]. Through a manifold of signaling pathways, changes in gene expression occur and result in the production of cytokines and T cell proliferation, among other factors involved in a pro-inflammatory response. A majority of these changes in gene expression are initiated by the transcription factors NFAT, NF-
κB, and AP-1 [83]. Overall, TCR/pMHC engagement induces T cell activation through the CD3 complex, signaling, and a pro-inflammatory response.

CD4 and CD8 co-receptors can affect TCR/pMHC engagement and T cell activation. Although not always the case, it is typically appreciated that CD8+ T cells mediate a cytotoxic T cell response through recognition of peptide in the context of MHC class I, while CD4+ T cells mediate a helper T cell response through recognition of peptide in the context of MHC class II [80]. The CD4 and CD8 glycoproteins play an important role in T cell activation. These co-receptors function by stabilizing the interaction between the T cell and antigen presenting cell via the MHC on the antigen presenting cell [85]. This stabilization can also have an impact on the affinity of the interaction. Additionally, it has been demonstrated that the cytoplasmic tails of CD4 and CD8 help recruit Lck to the CD3 signaling complex [91, 92]. Therefore, CD4 and CD8 are important co-receptors in T cell activation due to their dual functions.

The CD3 signaling complex is important for both TCR expression and T cell function. T cell activation and further TCR signaling in response to antigen recognition is essential to initiate an immune response. However, there are numerous factors within the TCR/pMHC interface that can impact the downstream T cell response. These factors and their potential impact on T cell function will be described in the next section.

**T cell Function**

T cells play a critical role in the adaptive immune response and cell-mediated immunity. T cells differentiate into a variety of subtypes dependent upon their function. These subtypes commonly include: effector, cytotoxic, helper, memory, regulatory,
natural killer, mucosal associated invariant, and gamma delta. Cytotoxic and helper T cells are the focus of this dissertation and therefore, will be discussed in further detail. The two major T cell subtypes examined in this dissertation are helper T cells and cytotoxic, or killer T cells [93]. CD4+ T cells are generally classified as helper T cells although, but they have been shown to have cytotoxic abilities [94, 95]. Helper T cells are important for directing an effector response and immune cell differentiation [96]. Secreted cytokine patterns further categorize T helper cells into two major categories, either Th1 or Th2, but the production of these cytokines are not mutually exclusive [95]. Other categories include Th17, Th22, and Th9. Cytokines important to the studies completed in this dissertation and their basic function are shown in Table 1. Th1 responses are important for cell-mediated immunity and are generally classified by IFN-γ and TNF-α secretion. Th2 responses are important for humoral-mediated immunity and are generally classified by IL-4, IL-5, and IL-13 secretion [96]. IL-2 has been shown to be produced in both Th1 and Th2 responses [97]. In summary, T cells are capable of secreting numerous different cytokines that affect an immune response and the patterns of cytokine secretion can categorize T cells into various T cell subclasses.

CD8+ T cells are generally classified as cytotoxic or killer T cells. These cells are important for the clearance of cells infected with intracellular pathogens as well as cancers [98]. Upon engaging with pMHC on a target cell, CD8+ T cells secrete large amounts of IFN-γ and TNF-α [99]. Secretory vesicles within the T cell, release perforin and granzyme which mediate death of the target cell [100]. Cell-mediated targeted cell death can also occur via the Fas/Fas ligand pathway.
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<tr>
<th>Cytokine</th>
<th>Function</th>
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<tr>
<td>IFN-γ</td>
<td>Activates T cells, NK cells, and macrophages, increase HLA expression on tumors, increases chemokine secretion [1, 96]</td>
</tr>
<tr>
<td>IFN-α</td>
<td>Involved in viral infections, increases HLA class I expression on tumors, enhances dendritic cell maturation [1, 101]</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Initiates pro-inflammatory innate immune response, induces fever, cell death, and hemorrhagic necrosis of tumors [1, 102]</td>
</tr>
<tr>
<td>IL-2</td>
<td>Stimulates T cell proliferation, activation, and memory differentiation [97]</td>
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<tr>
<td>IL-4</td>
<td>Induces expansion of Th$_2$ cells, activates B cells, enhances IgG and IgE production [96, 103]</td>
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<tr>
<td>IL-5</td>
<td>Stimulates antibody production, enhances proliferation of eosinophils, drives allergic type inflammatory responses [96, 104]</td>
</tr>
<tr>
<td>IL-6</td>
<td>Enhances antibody production and cytotoxic T cell differentiation, inhibit T regulatory differentiation [105-107]</td>
</tr>
<tr>
<td>IL-12</td>
<td>Induces expansion of Th$_1$ cells, enhances CD8$^+$ T cell activation, proliferation, and survival [1, 108]</td>
</tr>
<tr>
<td>IL-13</td>
<td>Facilitates B cell activation, promotes mucus production, mediator of allergic asthma, can inhibit production of pro-inflammatory cytokines [96, 109]</td>
</tr>
<tr>
<td>IL-15</td>
<td>Stimulates T cell and NK cell activation and proliferation, enhances survival of memory T cells [1, 110]</td>
</tr>
<tr>
<td>IL-17</td>
<td>Promotes recruitment and activation of innate cells, enhances B cell function, induces pro-inflammatory cytokine production [96, 111]</td>
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Table 1. Cytokines Discussed in this Dissertation and their Basic Function.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Function Description</th>
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<tr>
<td>IL-22</td>
<td>Regulates autoantibody production, promotes tissue regeneration [96, 112]</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Stimulates stem cells to produce granulocytes, increases dendritic cell and macrophage activation [1, 113]</td>
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Herein, Fas ligand, expressed on activated T cells, binds to Fas expressed on the target cell and causes cell death through initiated signaling cascades [114]. Following antigen encounter, T cells can expand and differentiate into effector and memory cells [115]. Additional T cell subclasses are characterized by their level of differentiation. Naïve T cells differentiate into stem cell memory T cells; central memory T cells; effector memory T cells; and effector T cells. Memory and proliferation decrease with differentiation, while effector function increases with differentiation [93]. These T cell subsets are commonly distinguishable by different surface markers. In CD8\(^+\) T cells, these surface markers include L-selectin (CD62L), CCR-7, CD45RO, and CD45RA [116, 117]. Specifically, naïve T cells are CD62L\(^+\)CCR7\(^+\)CD45RA\(^-\)CD45RO\(^-\), stem cell memory T cells are CD62L\(^+\)CCR7\(^+\)CD45RA\(^+\)CD45RO\(^+\), central memory T cells are CD62L\(^+\)CCR7\(^+\)CD45RA\(^-\)CD45RO\(^-\), effector memory T cells are CD62L\(^+\)CCR7\(^+\)CD45RA\(^+\)CD45RO\(^+\), and effector T cells are CD62L\(^+\)CCR7\(^+\)CD45RA\(^+\)CD45RO\(^+\) [93]. In CD4\(^+\) T cells, these surface markers include CD25, CD45RA, CD45RO, and CD127. Specifically, naïve T cells are CD25\(^-\)CD127\(^+\)CD45RA\(^+\)CD45RO\(^-\), central memory T cells are CD25\(^+\)CD127\(^-\)CD45RA\(^+\)CD45RO\(^+\), effector memory T cells are CD25\(^-\)CD127\(^+\)CD45RA\(^-\)CD45RO\(^-\), and effector T cells are CD25\(^-\)CD127\(^-\)CD45RA\(^+/-\)CD45RO\(^+/-\).
Many different factors have been described to be important in inducing memory differentiation such as strength and duration of TCR/pMHC interaction [118], help from CD4+ T cells and dendritic cells [119-121], natural killer group 2 member D (NKG2D) expression [122], and various cytokines and chemokines [123-127]. Memory T cells have been shown to rapidly re-express cytokines and lytic machinery. This hyper-responsive state allows them to rapidly respond to re-encountered antigen, which differs from naïve T cells [128-130]. More recently, tissue resident memory T cells have been identified and are characterized by their non-circulating nature and remain in peripheral tissue sites [116]. Central memory T cells continuously recirculate throughout the blood and lymphoid organs, such as the spleen and lymph nodes. Conversely, effector memory T cells generally traffic to non-lymphoid tissues [131]. These different memory T cells and further, memory subtypes, have been implicated to be important in immunotherapy. For instance, CD4+ and CD8+ central memory T cell subsets have been demonstrated as superior to effector memory T cell subsets in terms of activation, expansion, and persistence in vivo [132-135]. Strategies for generating these T cell subsets include purification on surface markers and modulation with cytokines [93, 132, 136, 137]. In conclusion, numerous T cell subsets exist and demonstrate unique functional phenotypes to elicit a critical role in adaptive immunity.

**TCR Affinity**

Affinity is a significant factor that shapes an immune response both in regards to antibodies and TCRs. One way in which TCRs and antibodies differ, is the fact that antibodies can undergo affinity maturation. Affinity maturation is the process in which antibodies increase their affinity throughout an immune response [138, 139]. Gene rearrangement of immunoglobulin V, D, and J segments occurs during B cell
development to generate a repertoire of B cells that express diverse antigen receptors. However, upon recognition of antigen, B cells undergo clonal expansion and affinity maturation [140]. B cells traffic to the germinal centers of lymphoid follicles and where they undergo somatic hypermutation in the CDR regions of the immunoglobulin genes which introduces single nucleotide substitutions to enhance antibody affinity, sometimes up to 100-fold higher, reaching the nM-pM range [141]. In summary, this process is imperative for antibody-mediated responses, however, affinity maturation does not exist, or is not required for T cell responses via their TCR.

TCRs do not undergo affinity maturation, nonetheless, TCR affinity has been implicated to have importance in a T cell response. Affinity is generally reported by the dissociation constant, $K_D$, a measurement used to describe the strength of the interaction between a TCR and a given pMHC complex [142]. Generally speaking, weakly self-reactive TCRs harbor affinities in the 10-100 μM range and high affinity TCRs that recognize foreign peptide fall in the 1-10 μM range [142, 143]. It has been generally appreciated that a higher affinity TCR will elicit a more robust T cell response; but more recently it is becoming increasingly apparent that TCR affinity is not always a direct correlate or adequate predictor of T cell function [144]. Higher affinity TCRs do not always elicit the most robust T cell response, as it has been demonstrated that TCRs with affinities around 1 μM or less result in decreased TCR signaling and T cell function [142, 145, 146]. Src homology 2 domain-containing phosphatase 1 (SHP-1) is an important negative regulator in TCR signaling by inactivating Lck. Studies show that SHP-1 is upregulated in an affinity dependent manner [142]. Overall, TCR affinity has
been demonstrated to be an important factor in T cell function and antigen recognition and is a key element of this dissertation.

The interaction between the TCR/pMHC impacts numerous kinetic parameters in addition to affinity. Another parameter is the dissociation rate ($k_{\text{off}}$) or half-life ($t_{1/2}$). Serial triggering is important to allow one pMHC complex to trigger multiple TCRs. It is estimated that one pMHC can trigger up to 200 TCRs [147]. Therefore, the optimal dissociation rate will be short enough to allow for other TCRs to bind the same pMHC, but long enough to allow for complete signaling [148]. This is critical in order to induce T cell activation despite the presence of very few pMHC complexes [149]. Evidence suggest that the $t_{1/2}$ can affect if the TCR behaves as an agonist, partial agonist, or antagonist [147]. This has been shown to alter patterns of ITAM phosphorylation and ZAP-70 activation. For example, lower affinity interactions may act as partial agonists and induce cytokine production but no proliferation [150, 151]. However, maximum cytokine production requires maintenance of the TCR and APC synapse while the release of cytotoxic granules is estimated to only require 1 to 3 pMHC complex interactions [151, 152]. In summary, the affinity of a TCR takes into account, as well as affects, multiple biophysical variables that successively influence the T cell response.

**Methods of Measuring TCR Affinity**

There are multiple methods that have been used to quantify TCR binding affinity. Some of these methods include surface plasmon resonance (SPR) [153], isothermal titration calorimetry (ITC) [154, 155], fluorescence resonance energy transfer (FRET) [156-158], fluorescence anisotropy [154, 159], the thermal fluctuation assay [160-162], tetramer off-rates [163-165], and the micropipette adhesion frequency assay [166].
Since SPR and the micropipette adhesion frequency assay are used in these studies, we will focus more in detail on these two methods for measuring TCR affinity.

The “gold standard” for measuring TCR binding affinity is generally via SPR. Briefly, either soluble TCR or pMHC, is secured to the sensor surface and the binding partner, either soluble TCR or pMHC, flows over the bound molecule. As binding occurs, mass accumulates on the sensor surface and increases the signal. When using SPR with immobilized TCR, the $K_D$ is equal to the concentration of free unbound pMHC when 50% of the immobilized TCR is bound [167]. TCR affinity and kinetic properties between soluble TCR and pMHC have most frequently been measured via SPR technology. These measurements provide information about the physical chemistry of the TCR/pMHC binding interaction. However, because three-dimensional (3D) measurements via SPR require soluble TCR and pMHC, they fail to account for aspects unique to membrane-bound proteins. The affinity measurement is limited to and dependent upon only the ligand-binding site. Therefore, TCR/pMHC binding kinetics measured via 3D SPR are advantageous for observing molecular TCR/pMHC properties, however, they have been demonstrated to be subpar for predicting T cell functional activities [168].

Systems have consequently been developed to study these membrane-bound interactions in a two-dimensional (2D) manner, and have been demonstrated to be better predictors of functional T cell outcomes. Unlike in SPR, these are cell to cell interactions. In one of these systems, the micropipette adhesion frequency assay, a red blood cell (RBC) serves as the APC and can be coated with pMHC via biotin-streptavidin coupling [169, 170]. Using a micropipette, a T cell and the pMHC coated
RBC are brought together and pulled apart via micromanipulation. Multiple parameters can be measured such as affinity, adhesion frequency, force, off-rate, and on-rate [162, 171]. Kinetic parameters of the TCR/pMHC measured in 2D have been shown in both CD4+ and CD8+ T cells to better correlate to T cell function than when measured in 3D [170, 172]. Specifically, one study using the 42F3 TCR (alloreactive TCR that recognizes HD-Ld presenting the peptide p2Ca933-940 of mouse 2-oxoglutarate dehydrogenase), demonstrated that 2D micropipette affinity measurements with multiple altered-peptide ligands better correlated to T cell potency than 3D SPR measurements. However, 3D SPR measurements better correlated with tetramer binding, eluting to the differences in TCR affinity using purified proteins or membrane-bound interactions [173]. In conclusion, due to the intrinsic factors of 3D and 2D affinity measurements, they can differentially correlate with T cell function. Affinity measurements obtained via SPR (3D) and via the micropipette adhesion frequency assay (2D) are included in this dissertation. Furthermore, how these measurements correlate to T cell function and cross-reactivity will be discussed in later chapters.

**T cell Cross-Reactivity**

Understanding T cell cross-reactivity is essential for my studies. The clonal selection theory suggested that B cells and T cells have specificity for only one antigen and it was doubtful they could recognize alternative ligands [174]. It was not until the 1990s that this paradigm was questioned and suggested highly improbable [175, 176]. The need for T cells to be inherently cross-reactive is now a fundamental concept in adaptive immunity and the idea of TCR binding degeneracy is well appreciated [177]. It has been estimated that there are less than 10^8 unique TCRs in the naïve T cell
population of humans [178]. Albeit, it has been estimated that an effective T cell repertoire must be capable of recognizing up to $10^{15}$ potential foreign antigens [177]. Therefore, the number of potential antigenic peptides exponentially surpasses the number of potential TCRs. Furthermore, estimations suggest a T cell clone must be able to recognize at least one million different peptides [176]. There are multiple reasons as to why a cross-reactive T cell population is advantageous. First, a cross-reactive T cell population allows for proficient immunity against an unlimited number of antigens via a limited number of T cells. Secondly, cross-reactivity reduces the potential for immune escape by pathogens because escape of recognition by one TCR may be recognized by another TCR. Thirdly, with cross-reactive T cells, fewer T cells are needed to scan for foreign antigen, which is both temporally and spatially advantageous [177]. Overall, it is well appreciated that T cells are cross-reactive and they need to be in order to maintain a comprehensive immune system and provide protection against the diverse pathogens we encounter.

The cross-reactivity of a T cell can be influenced by its TCR’s affinity with various ligands. Although exceptions exist, it is generally appreciated that higher affinity TCRs are more cross-reactive [179, 180]. It is believed that higher affinity TCRs can better withstand changes in the peptide structure and still allow for T cell activation. Conversely, in a lower affinity TCR, these changes in the peptide structure could result in reduced binding energy and thus, yield an interaction that is below the threshold of T cell activation [148]. How TCR affinity affects T cell cross-reactivity will be further discussed throughout this dissertation. In summary, T cells are cross-reactive and the level of cross-reactivity can be influenced by TCR affinity.
The Immune System and Cancer

The immune system is essential for controlling many malignant cells. It is estimated that a cell can experience over 20,000 DNA damaging events a day, but these are normally repaired [181, 182]. A malignant cell can have more than an estimated 11,000 mutations [183]. The immune system can detect and kill these malignant cells. This was first described by Burnet and Thomas in the cancer immunosurveillance hypothesis [184-186]. When a tumor grows to more than 2-3 mm, blood supply and stromal remodeling induces proinflammatory cytokines and initiates the recruitment of innate cells, such as dendritic cells, macrophages, and NK cells, to the tumor where they produce IFN-γ [187]. The recruited dendritic cells then take up tumor associated antigens and cross-prime T cells in lymph nodes [188]. Tumor associated antigens can include viral, mutated, differentiation, cancer germline, or overexpressed antigens [189]. Conveniently, the “Cancer Antigenic Peptide Database” provides a regularly updated list and characterization of numerous different tumor antigens [190]. Tumor antigen specific T cells then traffic to the tumor site and eliminate the tumor cells [191]. Overall, both innate and adaptive immune cells are important for the control of malignant cells.

The idea of cancer immunoediting was subsequently described to include the immune system’s role in both host protection and tumor sculpting. The immunoediting process has been further described in three phases: elimination, equilibrium, and escape [192]. After elimination, as described above, the equilibrium phase consists of continuous elimination of the tumor, while selecting for tumor cell variants that can evade the immune response. Escape occurs when the tumor cell variants expand
and/or metastasize due to loss of control by the host immune system [191]. Cancers have developed numerous mechanisms to escape recognition by the immune system [191]. Mainly, this is achieved by creating a “cold” immunosuppressive tumor microenvironment [193]. To name a few of these mechanisms, tumors can downregulate MHC expression, mutate antigen processing pathways, or lose expression of, or mutate, the targeted antigen [194]. Tumors can express checkpoint molecules to suppress T cell function. To generate an immunosuppressive environment, tumors can recruit regulatory T cells, myeloid-derived suppressor cells, and type II macrophages [195]. Overall, identification of these numerous immunosuppressive mechanisms elicited by tumor cells, has been important in expanding the field of tumor immunology [196].

**History of Immunotherapy**

Scientists have been searching for decades to find new ways in which the host’s immune system can be exploited to treat cancer. In the 19th century, it was first observed that erysipelas infection aided tumor regression [197]. By 1959, it was shown that Bacillus Calmette-Guerin (BCG), an attenuated live bovine tubercle Bacillus-based vaccine for tuberculosis, could inhibit tumor growth in mice [198]. This was the first immunotherapy based treatment utilized for cancer treatment in 1970 [199]. Subsequently, IL-2 was discovered upon its ability to activate and expand T cells [200]. It was further demonstrated that administration of recombinant IL-2 (rIL-2) to tumor bearing mice elicited regression of pulmonary metastases and subcutaneous tumors [201]. These exciting results were quickly translated into the clinic. In the first six cancer patients treated with high dose IL-2, three patients had objective responses [202].
However, since not all patients responded, the combination of cellular immunotherapy and IL-2 treatment was investigated. It was demonstrated that peripheral blood mononuclear cells (PBMCs) cultured with IL-2 could generate lymphocyte-activated killer (LAK) cells and could then kill tumor cells *in vitro* [203-205]. *In vivo* models combining rIL-2 treatment with adoptive transfer of LAK cells into tumor bearing mice demonstrated antitumor activity if treated prior to tumor vascularization [206, 207]. Following these results, the first cellular immunotherapy combined high dose IL-2 with LAK cells [208]. LAK cells are made up of NK cells and NK T cells, however, their antitumor efficacy with high dose IL-2 was as effective as high dose IL-2 alone [209-211]. Since then, IL-2 has been and is currently used in combination with many forms of immunotherapies in many clinical trials [212].

TIL was an attempt to improve upon the responses obtained with IL-2 or LAK cells in cancer therapies. TIL have been found in melanoma lesions for over forty years [213]. It was later demonstrated that culturing TIL *ex vivo* in high amounts of IL-2 could restore their proliferation and lytic function [214]. Objective responses were observed in about 31% of melanoma patients receiving autologous cell transfer of *ex vivo* TIL expansions and IL-2 treatment [215]. To improve upon this, these therapies were given in conjunction with lymphodepletion, where objective responses rose to 55% in melanoma patients [212, 215-218]. Host lymphodepletion has been demonstrated to be important to enhance the efficacy of adoptively transferred cells for a few reasons. Specifically, lymphodepletion can create space for the transferred cells and deplete regulatory T cells [219, 220]. A 72% response rate was later observed in metastatic melanoma patients treated with 12 Gy of total-body irradiation prior to TIL and IL-2
treatment [221]. In summary, the observation of TILs present in the tumor lesions has eventually supported the notion of utilizing T cells for cancer targeted therapies [222].

Aside from IL-2, LAK, and TIL, numerous other forms immunotherapies have subsequently been investigated. These include cytokines, chemokines, dendritic cell-based vaccines, antibodies, and gene-modified cells. Other cytokines used in clinical trials include TNF-α, IFN-γ, IFN-α, IL-15, and GM-CSF [1]. Efficacies of single agent cytokine therapies ranged between 5% and 43% of patients exhibiting partial responses and 13% and 42% of patients exhibiting complete response, however, many treatments exhibited toxicities [1]. One open clinical trial is treating patients with TIL transduced to express CXCR2, with the expectation that CXCR2 will enhance T cell trafficking to the tumor [1, 223]. Peptide and peptide pulsed dendritic cell vaccination strategies have not been successful in melanoma, with only 2.6% of patients having an objective response [224, 225]. This was most likely due to the observed functional inabilities of melanoma reactive T cells in patients [226]. Albeit, Sipuleucel-T, a dendritic cell vaccine, has been FDA approved for treatment of castrate-resistant metastatic prostate cancer [227].

Furthermore, vaccinations targeting patient specific neo-antigens induced expansion of neo-antigen specific T cells and protected against tumor reoccurrences [228-230]. Neo-antigens have become an attractive focus for research, as it has been demonstrated that tumor mutation burden can be a strong determinant of responses with immunotherapies [231-235]. Targeting neo-antigens is advantageous due to their exclusive expression on tumor cells, and thus, could reduce the potential for on-target, off-tumor cross-reactivity [236, 237]. Overall, ongoing research may provide evidence
on the role of vaccination in T cell responses and anti-tumor immunity, especially in the targeting of neo-antigens.

In the last three decades, advancements have been made in identifying suppressive receptors that inhibit T cell responses. These receptors are classified as checkpoints and include programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte associated protein 4 (CTLA-4), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and lymphocyte activation gene-3 (LAG-3). CTLA-4 was first identified in 1988 and by 1996 it was demonstrated that blocking CTLA-4 could enhance anti-tumor responses in mice [238-240]. In subsequent clinical trials, response rates ranged from 16.2% to 28.5% of patients and responses were often durable, lasting more than 34 months [241-243]. The checkpoint blockade mAb targeting CTLA-4 was the first immunotherapy drug to become FDA approved in 2011 for the treatment of melanoma [240, 244-247]. Overall, CTLA-4 mAb has demonstrated success as a single agent in immunotherapy.

The second checkpoint, PD-1, was identified and cloned in 1992 and 1994, respectively [248, 249]. It was later demonstrated that PD-1 ligand (PDL-1) expression on tumor cells promoted tumor escape via T cell suppression in mice [250, 251]. Furthermore, in mouse models, tumor growth could be suppressed using an anti-PD-L1 mAb [251, 252]. In subsequent clinical trials, up to 40% of patients had objective responses and responses were durable, lasting more than one year [245, 253, 254]. Consequently, the checkpoint blockade mAb targeting PD-1 or PD-L1 has also been FDA approved [253, 255]. Overall, checkpoint blockade antibodies demonstrated
success as single agent therapies and thus, remain a promising area of research for combination immunotherapies.

A robust T cell response is important for the success of cancer immunotherapies. Therefore, further improvements in T cell therapies have been developed using TCR or CAR gene-modified T cells to redirect the specificity of T cells. In the 1980’s, scientists explored methods of combining the functional response of a T cell with the diversity of antibody recognition. As a result, CARs were developed to consist of an extracellular single chain variable fragment linked to an intracellular signaling domain [256]. The first described CAR contained the Fc receptor (FcR) γ chain signaling domain, and subsequent CARs contained the zeta chain of the CD3 signaling complex [256, 257]. The CD3ζ chain contains three ITAMs compared to FcRγ’s single ITAM. Succeeding CARs have been developed over the years with varying signaling and stimulatory components. Specifically, in addition to the CD3ζ domain, second generation CARs implemented another costimulatory domain (such as CD28, 4-1BB, or OX40) and third generation CARs implemented two additional costimulatory domains [257-261]. A schematic of the different generations of CARs is depicted in Figure 1. CAR genes (and also TCR genes) are commonly inserted into T cells via viral vectors. Numerous different viral vectors have been utilized to insert genes into T cells. Some of these include adenoviral, retroviral, lentiviral (technically retroviral), and poxviral [262]. Retroviruses are commonly used due to their ability to integrate into the host cell’s genome [263, 264]. Specifically, lentiviral vectors and γ-retroviruses, generally based on murine leukemia virus (MLV), are frequently used in gene therapy [262].
CARs include an extracellular single chain variable fragment comprised of variable light (V<sub>L</sub>) and heavy (V<sub>H</sub>) chains linked via hinge. Intracellular domains, linked via hinge, in first generations CARs included either FcRγ or CD3ζ chain signaling domains. Second generation CARs included an additional costimulatory domain. Third generation CARs included two costimulatory domains.

In summary, various viral vectors have been utilized to introduce various generations of CARs into T cells to redirect T cell specificity towards the antigen of choice.

A wide variety of CARs have been developed to target an array of antigens both in vitro and in vivo. Some of these targets include antigens expressed on glioblastoma, neuroblastoma, melanoma, hematologic malignancies, prostate cancer, breast cancer, colorectal cancer, and ovarian cancer [258]. CARs are advantageous due to their ability to recognize antigen in an MHC independent manner. However, adverse events in clinical trials have been documented due to “on-target, off-tumor” reactivity, tumor lysis syndrome, and cytokine storm [258]. CAR T cells have exhibited varying levels of clinical success, albeit, the greatest success with CAR T cells has been exhibited in recent years with the treatment of refractory B-cell leukemia [265, 266]. The first clinical
trial using the CD19-targeting CAR resulted in objective responses in 57% of patients [265, 266]. These rates have since increased up to almost 80% complete response rates in patients [267, 268]. Consequently, the use of autologous CAR T cells for the treatment of large B-cell lymphoma was FDA approved in 2017 [265, 269, 270]. Overall, the use CAR T cells for treatment of B-cell malignancies has been a significant breakthrough in the field of immunotherapy and CAR T cells remain a promising area of research in terms of gene-modified T cells.

TCR gene-modified T cells have also shown success in the clinic and remain a significant area of research in addition to CAR T cells. TCR gene-modified T cells are the focus of this dissertation and thus, will be described in more detail. As TIL therapies exhibited success in many patients, researchers began to identify and isolate tumor-reactive TCRs. The first melanoma specific TCRs were cloned in 1994 and 1995 [271, 272]. Subsequently, the use of TCR gene-modified T cells for adoptive cell transfer moved into clinical trials. A graphical schematic of the process of using TCR gene-modified T cells for adoptive T cell transfer therapy is depicted in Figure 2 (this process is the same for CAR T cells, except CAR genes are inserted into a viral vector instead of TCR genes). In the first clinical trial, using a TCR targeting a melanoma associated antigen, 2/15 patients had objective clinical responses and engraftment of the introduced T cells [273]. In clinical trials thus far, the use of TCR gene-modified T cells has yielded efficacies in up to 55% of treated patients [1, 274, 275]. Overall, the use of TCR gene-modified T cells has become increasingly widespread in immunotherapies, with advancements in utilization as a single agent therapy, as well as in combination therapies.
Figure 2. Graphical Representation of Generating TCR Gene-Modified T cells for Adoptive T cell Transfer Therapy. Tumor reactive T cell clones are isolated and expanded from the PBMCs or TIL of a patient with cancer. TCR alpha and beta chain genes that are reactive against tumor associated or viral antigens are identified and cloned into a viral vector. Packaging and producer cell lines are utilized to make high titer virus. Patient activated peripheral blood T cells are transduced with viral supernatant, expanded, and administered back to the patient. The specificity of these circulating T cells is now redirected toward the specific tumor or viral antigen resulting in anti-tumor immunity.

Adoptive T cell therapy and the use of TCR gene-modified T cells have revolutionized the field of immunotherapy through their ability to target specific tumor or viral antigens of choice [276]. With all the success of this treatment, there are also potential drawbacks in utilizing TCR gene-modified T cells. First, introduction of another TCR into the T cell allows for the potential of αβ TCR chain mispairing between the introduced and endogenous TCR chains. The formation of mixed dimers can lead to new TCR specificities and result in autoimmunity. This phenomenon has been demonstrated in murine models [277, 278], but has never been proven to have occurred in clinical trials. Strategies have been implemented to enhance proper TCR chain pairing by making modifications to the TCR genes. These strategies include: addition of
another disulfide bond, replacing human constant regions with murine constant regions, codon optimization, leucine zipper fusion proteins, and single chain TCRs [94, 279-286]. In vitro evidence suggested modifying the TCR genes to express murine constant regions or leucine zipper fusion proteins resulted in enhanced proper TCR chain pairing and T cell function [94]. Overall, one of the challenges associated with the introduction of a new TCR is TCR chain mispairing. TCR chain mispairing has the potential to cause autoimmunity, and strategies to enhance proper TCR chain pairing have been investigated.

Properly paired introduced TCRs can also cause potential cross-reactivity of the TCR gene-modified T cells. Specifically, TCR gene-modified T cells can recognize the on-target antigen on normal tissue or an off-target antigen on normal tissue [287]. On-target, off-tumor antigen recognition has been described in clinical trials using non-modified TCRs. Specifically, this occurred where the TCR target was a melanoma antigen that is also expressed in the ear, eye, and normal melanocytes, and where the TCR target was a colorectal cancer antigen that is also expressed in epithelial cells throughout the gastrointestinal tract [288-290]. The clinical implications of this drawback, moreover when modifying the TCR, will be discussed in the next section. The focus of this dissertation will include a novel strategy proposed to enhance the efficacy and safety of TCR gene-modified T cells for adoptive T cell transfer.

Adverse Events Observed in Clinical Trials using Affinity Enhanced TCRs

Despite the success seen in clinical trials, the use of TCR gene-modified T cells does not elicit an anti-tumor response in every patient. TCRs that target self-antigens generally harbor a low affinity as a result of negative selection in the thymus. Therefore,
TCR affinity enhancement has been utilized as a strategy to enhance the anti-tumor efficacy of TCR gene-modified T cells. Specifically, yeast or phage display is frequently utilized as a method for affinity enhancement [291-296]. However, random mutation through yeast or phage display can lead to unanticipated off-target cross-reactivity [142]. Adverse events have been observed in clinical trials where autologous affinity enhanced TCR gene-modified T cells were used and have been attributed to cross-reactivity. In summary, TCR affinity enhancement can augment the anti-tumor response, but also has the potential to cause off-target autoimmunity in patients.

There are two clinical trials where affinity enhanced TCRs were utilized in TCR gene-modified T cells and resulted in unexpected patient deaths. An affinity enhanced HLA-A2 restricted TCR that targeted MAGE-A3 was used to treat nine cancer patients. This TCR was previously affinity enhanced by mutating a residue that improved T cell reactivity in CD8+ T cells and allowed for recognition in CD4+ T cells. This TCR was chosen from 85 variant TCRs containing single or multiple amino acid substitutions in the CDR3 region of the TCR alpha chain [297]. 4/9 patients exhibited neurological toxicity and two of these patients subsequently died. Further investigation indicated that MAGE-A12 and possibly MAGE-A1, MAGE-A8, and MAGE-A9 were expressed in the brain. In this case, the reactivity against these other MAGE-A targets was known, but their expression in the brain was not. It is unclear as to if the WT TCR would have resulted in the same observed neurological toxicity. These adverse events can be attributed to an on-target, off-tumor cross-reactivity of the introduced TCR gene-modified T cells [5].
In the second case, an affinity enhanced HLA-A1 restricted TCR that targeted MAGE-A3 was affinity enhanced via phage display through four substitutions in the CDR2 region of the TCR alpha chain. Herein, 2/2 treated patients died from cardiac toxicity four or five days after TCR transduced T cell infusion, respectively. Further analysis indicated that the TCR gene-modified T cells had trafficked to the heart and histological results were consistent with immunologically mediated damage. This is an example of off-target, off-tumor cross-reactivity [6]. It was later determined that the affinity enhanced TCR recognized the Titin protein expressed on cardiomyocytes, whereas recognition of Titin was not conferred in T cells expressing the WT TCR. The MAGE-A3 and Titin peptides differ in sequence in four of nine residues, including the core, yet the affinity enhanced TCR recognized both peptides in the context of MHC with almost identical conformation of the CDR loops [298, 299]. This is an example of how high affinity TCRs can tolerate alterations in the peptide structure [148]. Pre-clinical screening for potential cross-reactivity of the affinity enhanced TCR was completed using only a limited number of off-target tissues. However, Titin is only expressed in beating cardiomyocytes derived from induced pluripotent stem cells and the epitope is not homologous in the mouse [298, 299]. These results exemplify the potential dangers of non-specifically affinity enhancing a TCR without extensive pre-clinical screening.

Both of these described clinical trials demonstrated the potential dangers in using affinity enhanced TCRs, especially non-specific enhancement, even when done so with an originally low affinity TCR. However, despite the risks involved in using an affinity enhanced TCR, one affinity enhanced TCR was used in patients that did not result in adverse events [300-304]. This NY-ESO-1 targeting TCR contained two mutations in
the CDR3 region of the TCR alpha chain that enhanced T cell reactivity [304]. Overall, these results indicated that TCR affinity enhancement does not always lead to off-target cross-reactivity.

Even though affinity enhanced TCRs did not result in adverse events in every clinical trial, it is evident that we need more advanced and meticulous strategies in order to enhance the anti-tumor responses of TCR gene-modified T cells while still sustaining safety for treated patients. The purpose of this dissertation is to address that current obstacle in the field.

**MART-1 and DMF5 TCR**

One subclass of tumor associated antigens includes differentiation antigens. Many differentiation antigens have been identified for their frequent expression in melanomas. Some of these include tyrosinase [305, 306], premelanosome protein (PMEL or gp100) [307-309], and tyrosinase related protein 1 and 2 (TRP1 and TRP2) [310, 311]. Herein, we focus on the melanoma differentiation antigen, melanoma antigen recognized by T cells 1 (MART-1). MART-1 is lineage-specific protein found in melanocytes and is important for melanosome maturation [312].

MART-1 was first identified in 1994 by cDNA expression cloning [313]. It was recognized by a melanoma patient’s HLA-A2 restricted TIL. MART-1 mRNA was found in 11 out of 14 melanoma lines. Furthermore, expression of MART-1 was confirmed in retinal tissue, but no other normal tissue lines [313]. Subsequent studies show MART-1 expression in up to 90% of melanomas [314]. MART-1 epitopes have been recognized in the context numerous different HLAs, expressed on both MHC class I and MHC class II [315-320]. Expression of this tumor associated antigen has been linked to significantly
longer overall survival in melanoma patients [321]. In summary, MART-1 has been a frequently targeted melanoma antigen for therapy due to its recurrent, and sole expression on melanocytes and melanomas.

MART-1 is a unique antigen because high frequencies of MART-1 reactive T cells are found in both cancer patients and normal donors [322-324]. Both the MART-1(27-35), nonameric, and MART-1(26-35), decameric, peptides are recognized by MART-1 reactive T cells [324, 325]. The MART-1(27-35) epitope (AAGIGILTV) has been regarded as an immunodominant epitope [326]. MART-1 anchor modified epitopes have been generated and are discussed in further detail in Chapter Three. It has been hypothesized that the high frequency of MART-1 reactive T cells in the blood of normal donors and cancer patients is due to epitope mimicry. This is a result of T cells being primed by a foreign or pathogen derived antigen and subsequently reacting with a formerly ignored self-antigen [327]. The MART-1 nonameric epitope is extremely hydrophobic, with only one neutral threonine at position eight. Transmembrane domains and leader peptides of proteins are also very hydrophobic. Furthermore, studies show that cytosolic proteins with central hydrophobic core are the major substrates of proteasomal degradation. Hence, why it is well appreciated that hydrophobicity is strongly correlated to immunogenicity [68]. It has been shown that MART-1 derived TIL cultures and MART-1 specific T cell clones can lyse peptides in the context of HLA-A2 that have some degree of homology with MART-1 [327, 328]. Since MART-1 reactive T cells are uniquely found in healthy donors, most likely due to epitope mimicry, we thought it would be an interesting antigen to target while studying the cross-reactive properties of TCRs.
Multiple MART-1 reactive TCRs have been used in clinical trials. With the use of autologous PBMCs engineered to express a MART-1 reactive TCR (unnamed), 2/15 patients demonstrated sustained objective responses [329]. Intra-tumoral injections of allogeneic T cells expressing the MART-1 reactive TCR, TIL 5 [271, 330], resulted in 1/15 patients demonstrating a partial response [331]. The use of another MART-1 reactive TCR, DMF4, resulted in objective responses in 13% of the patients and none of the patients exhibited normal melanocyte destruction in the ear or eye [329, 332]. Additionally, the MART-1 reactive TCR, DMF5, was used in a clinical trial where objective responses were observed [288, 332]. This HLA-A2 restricted DMF5 TCR was utilized in the studies completed in this dissertation and thus, will be described in further detail.

The DMF5 TCR was isolated from a MART-1 reactive TIL clone from a melanoma patient, the same patient from which the DMF4 TCR was cloned. This TCR is classified as a high affinity/avidity and CD8 independent TCR [332]. The DMF5 TCR was utilized in a clinical trial using autologous TCR gene-modified T cells for adoptive cell transfer in 20 melanoma patients. Objective responses were seen in 30% of the patients. Albeit, 17/20 patients exhibited melanocyte destruction in off-tumor tissues, specifically the eye, ear, and skin. Some of the patients experienced these adverse events up to grade 3 toxicities. This is an example of an on-target but off-tumor cross-reactivity of the introduced TCR gene-modified T cells. Additionally, some of the patients exhibited off-tumor melanocyte destruction in the absence of any tumor rejection. The DMF5 TCR has a higher affinity than the DMF4 TCR. This possibly explains why the percentages of objective clinical responses were higher with the DMF5
TCR. Furthermore, this possibly explains the observation of off-tumor melanocyte destruction with the DMF5 TCR, but not the DMF4 TCR. This clinical trial indicated that T cells expressing the DMF5 TCR can have a therapeutic anti-tumor response in melanoma patients [288]. Overall, due to the high level of epitope mimicry observed with MART-1 “like” epitopes and the clinical relevance of the DMF5 TCR, we believed the MART-1/DMF5 TCR model was fitting for understanding TCR specificity and cross-reactivity.

**Novel Structure-Guided Approach**

As mentioned previously, one of the strategies for enhancing the anti-tumor response of TCR gene-modified T cells is by TCR affinity enhancement via yeast or phage display. It is possible that use of an affinity enhanced TCR can result in unanticipated cross-reactivity or recognize unpredicted targets, and thus, modified TCRs for adoptive transfer of TCR gene-modified T cells need to be addressed more carefully.

The process of introducing mutations into TCRs to alter affinity and T cell specificity is not a new concept. There are many examples in different TCRs where introduced mutations affected affinity, binding kinetics, antigen specificity, and cross-reactivity. For example, CDR1, CDR2, and CDR3 substitutions in the 2C (alloreactive TCR that recognizes alpha-ketoglutarate dehydrogenase in the context of H-2L^d and syngeneic recognition of SIYR peptide in the context of H2-K^b) TCR’s alpha and beta chains resulted in varying levels of altered-peptide ligand recognition and tetramer binding. TCR mutations also altered the recognition of the targeted peptide in CD4^+ and CD8^+ T cells [333, 334]. More specifically, high affinity mutant TCRs demonstrated
higher binding to altered-peptide ligands compared to the WT TCR [335]. One TCR mutation enhanced affinity for the targeted ligand 1,000-fold higher than the WT TCR. There was a 5- to 10-fold increase in T cell potency against the targeted ligand, but CD8+ T cells expressing this high affinity 2C TCR were autoreactive [179, 333]. In another example, high affinity 3.L2 TCR (HI-Ek restricted, recognizes β-chain of mouse hemoglobin) variants were also more degenerate in their recognition of altered-peptide ligands, both in potency and in the number of recognized targets, compared to the WT TCR [336]. In a third example, single CDR3 TCR alpha and beta chain mutations were introduced into the 1MOG9 TCR (HI-Ab restricted, recognizes myelin oligodendroglial glycoprotein) based on the importance of TCR CDR3 loops in peptide binding [337-340]. Herein, high affinity TCRs did enhance targeted antigen potency and confer co-receptor independence compared to the WT TCR. However, the introduction of mutations in the 1MOG9 TCR also led to new recognition of self-peptides [341]. Lastly, yeast libraries have been utilized as a strategy in in vitro directed evolution, specifically, for intentionally changing the antigen specificity of the A6 TCR (HLA-A2 restricted, recognizes Tax) [342]. Other studies have claimed to have enhanced targeted antigen specificity with TCR mutants generated via phage display, but an examination of cross-reactivity and T cell recognition of altered-peptide ligands was marginal or absent [343, 344]. Despite the many examples of high affinity TCR variants resulting in new recognition of altered-peptide ligands, this is not always the case. For example, using a WT1-specific TCR, two high affinity TCR clones with CDR3 TCR alpha chain mutations were selected from yeast display libraries. The affinity enhanced TCRs demonstrated enhanced targeted T cell reactivity in CD4+ and CD8+ T cells compared to the WT TCR.
Additionally, mice that were injected with T cells expressing the WT or affinity enhanced TCRs with irradiated peptide pulsed splenocytes, exhibited no signs of autoimmunity, and expansion of TCR transduced T cells was similar among the different groups. Pathology after two to three weeks indicated no T cell-mediated tissue damage within the different treatment groups. These results provided further evidence that not all affinity enhanced TCRs will cause autoimmunity in vivo [345]. In another example, single and dual mutations in the CDR3 TCR alpha chain and CDR2 TCR beta chain of the 1G4 TCR (HLA-A2 restricted, recognizes NY-ESO-1) enhanced affinity and targeted antigen potency compared to the WT TCR, but cross-reactivity or recognition of altered-peptide ligands was not addressed in these studies [304]. However, one of these modified 1G4 TCRs was safe when used in patients [300, 302]. In summary, many studies suggest TCR mutations in the CDR1, CDR2, and CDR3 regions impact TCR affinity and T cell specificity. Specifically, TCR mutations can alter binding kinetics, antigen recognition in CD4+ and CD8+ T cells, and cross-reactivity. The potential off-target reactivity as of result of TCR mutations, implicates the need for better TCR mutational methods and approaches.

Herein, in collaboration with the Baker’s laboratory, we have developed and implemented an approach to improve specific antigen reactivity and reduce off-target cross-reactivity by modifying the TCR using structure-guided mutations. More specifically, we used the crystal structures of the TCR/pMHC to implement the structure-guided design as a novel component versus inducing random mutation in the TCR. This strategy involves the combinatorial idea of “positive and negative design”. “Positive and negative design” has been utilized in other fields as a method to alter
binding specificity [346-348]. Herein, as a way to enhance antigen specificity, the positive design will introduce mutations in the TCR that enhance binding to the peptide by introducing favorable interactions. The negative design will introduce mutations in the TCR that weaken or eliminate binding of TCR residues with the MHC by weakening existing favorable interactions [349]. Negative mutations could also be translatable to other TCRs if they are made at conserved MHC contacting residues in the TCR and thus, would be common to most presented peptides. It is imperative to recognize the importance of the negative mutations as a way to offset the positive mutations, as introducing only favorable interactions with the peptide could allow for unwanted effects on T cell specificity. Instances of this occurring with different TCRs has already been observed and examples have been described above. Even though negative TCR mutations would reduce the TCR affinity, the enhanced binding towards the specific peptide would persist [349]. The combination of these positive and negative TCR mutations is hypothesized to enhance antigen specificity while simultaneously reducing potential cross-reactivity. Although these mutations will affect the affinity of the TCR, the idea is to redistribute the free binding energy and not solely focus on enhancing TCR affinity. This redistribution of free binding energy would allow for the TCR to have an increased focus on the peptide and a decreased focus on the MHC [350]. This novel structure-guided design strategy is used in this dissertation as an approach to fine-tune TCR specificity and to generate a more focused DMF5 TCR, based on the solved crystal structure of the TCR/pMHC [351].
Concluding Remarks

T cells play a significant role in the adaptive immune response. T cell-mediated immunity is dependent upon TCR recognition of pMHC. In order to maintain an inclusive immune system, T cells must be cross-reactive. However, negative selection in the thymus is critical for eliminating highly autoreactive T cells. More recently, T cells have been harnessed in many forms of immunotherapies for their ability to kill tumor cells. Namely, the use of TCR gene-modified T cells has shown success in clinical trials. To improve the efficacy of TCR gene-modified T cells, methods of TCR affinity enhancement have been utilized. These TCR modifications do not always cause harm to patients, but affinity enhanced TCR gene-modified T cells have resulted in lethal adverse events due to off-target cross-reactivity mediated by the introduced T cells. Consequently, the field needs more advanced and meticulous strategies in order to enhance the anti-tumor responses of TCR gene-modified T cells while still sustaining safety for treated patients. In these studies, in collaboration with the Baker lab, we sought to develop and utilize a novel structure-guided design approach to fine-tune the antigen specificity of TCRs.

Herein, we utilized the DMF5 TCR/MART-1 model. Specifically, our objective was to enhance MART-1 specificity while simultaneously reducing cross-reactivity. Overall, when altering TCRs for therapeutic use, biology and safety should be of the utmost importance and herein, we emphasize the importance of rigorous preclinical testing of modified TCRs and the need for advancement in modeling/prediction tools for protein interactions.
CHAPTER TWO
MATERIALS AND METHODS

Cell Lines, Media, and Reagents

T2 [352, 353], HEK293GP [354-356], PG13 [357, 358], and Jurkat E6.1 cells [359] were obtained from the American Type Culture Collection (Manassas, VA). T2 cells are TAP deficient and therefore cannot load their own peptide onto MHC class I. The MHC becomes stable on the cell surface when loaded with exogenous peptide. T2 cells were used as stimulator cells for T cell functional assays. HEK293GPs are a human embryonic kidney packaging cell line that were made to express the retroviral gag and polymerase proteins. HEK293GP cells were used to produce high titer retrovirus by transient co-transfection using a retroviral vector and a plasmid containing the vesicular stomatitis virus (VSV) envelope. PG13 cells (based on Gibbon ape leukemia virus (GaLV)) are a retroviral producer cell line that when transduced with HEK293GP supernatant, will stably produce high titer retrovirus. Jurkat E6.1 cells are a CD4-CD8- human T cell lymphoblast used for TCR transduction and functional assays. The tumor cell lines used in T cell functional assays are listed in Table 2. All tumor lines were obtained from American Type Culture Collection (Manassas, VA) except MEL 624 and 624-28 [360], UOK131 [361], and SAUJ (Rick Childs, NCBI) [362] were obtained from the NIH Surgery Branch (Bethesda, MD).
Table 2. Tumor Cell Lines and Phenotypes. “+” indicates positive and “-” indicates negative.

<table>
<thead>
<tr>
<th>Name</th>
<th>Tumor/ Tissue</th>
<th>MART-1</th>
<th>HLA-A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEL 624</td>
<td>Melanoma</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MEL 624-28</td>
<td>Melanoma</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MEL A375</td>
<td>Melanoma</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SAUJ</td>
<td>Renal</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UOK131</td>
<td>Renal</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SKOV3</td>
<td>Ovarian</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CAPAN 1</td>
<td>Pancreas</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MDA 231</td>
<td>Breast</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SW480</td>
<td>Colon</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>HEPG2</td>
<td>Liver</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SKGT5</td>
<td>Esophagus</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>U251</td>
<td>Glioblastoma</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

All medium components were obtained from Corning Life Sciences (Corning, NY), unless otherwise noted. T2 and Jurkat E6.1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Carlsbad, CA). HEK293GP cells were maintained in complete medium consisting of Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS. PG13 cells were maintained in complete medium consisting of Iscove’s DMEM supplemented with 10% FBS. All tumor cell lines, except SAUJ cells, were maintained in complete medium consisting of Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS. SAUJ tumor cells were maintained in complete medium consisting of RPMI 1640 medium supplemented with 10% FBS.

**T cells**

All PBMC used in this study came from de-identified apheresis products purchased from Key Biologics (Memphis, TN). PBMCs were isolated from normal
healthy donors by Ficoll-Paque (General Electric, Fairfield, CT) density gradient centrifugation. Briefly, whole blood was diluted 1:2 in phosphate-buffer saline (PBS) (Mediatech, Inc., Manassas, VA), loaded onto Ficoll density gradients, and spun at 2,000 RPM for 30 minutes at room temperature (RT) without brake. The density of Ficoll is 1.077 g/mL, which allowed red blood cells to pass through the Ficoll and inhibited the passage of white blood cells. Buffy coat was collected and washed three times with PBS. All T cells were maintained in complete T cell medium consisting of RPMI 1640 medium supplemented with 10% FBS, 300 IU/mL recombinant human IL-2 (rhIL-2; Novartis Pharmaceuticals Cooperation, East Hanover, NJ), and 100 ng/mL recombinant human IL-15 (rhIL-15; Biologic Resources Branch, NCI, Frederick, MD) at 37°C in a humidified 5% CO₂ incubator.

**Peptides**

All peptides were obtained from Synthetic Biomolecules (San Diego, CA) and were HPLC purified at 95%. Peptides were stored at 10 µg/mL in 100% DMSO at -80°C.

**Retroviral Vector**

Our lab uses a modified SAMEN retroviral construct to introduce TCR genes into T cells [363]. The structure of this vector is shown in Figure 3. At the 5’ end of the vector is a human cytomegalovirus (CMV) long terminal repeat (LTR) promoter. This promoter allows for enhanced constitutive levels of transcription. Following this promoter is a splice donor and splice acceptor site for RNA splicing. Ψ (psi) is the packing signal. The TCR alpha gene and TCR beta gene are linked by a P2A self-cleaving peptide. This allows the alpha and beta chains to be synthesized in a 1:1 ratio.
A modified SAMEN retroviral vector was used to transfer TCR genes to alternate effectors. The retroviral vector used in this study contains a CMV promoter, splice donor (SD) and splice acceptor (SA), psi (ψ) packaging signal, TCR alpha (α) and beta (β) chains, CD34t, and long terminal repeats (LTR). The TCR α and β genes and the CD34t molecule are linked via P2A and T2A sequences, respectively. P2A and T2A are self-cleaving peptides cut into three separate proteins. CD34t is used as a marker for transduction.

Following the TCR beta gene is a T2A self-cleaving peptide followed by CD34t which is synthesized in a 1:1 ratio with the TCR chains. CD34t is a truncated CD34 molecule and consequently lacks its intracellular signaling domain [364]. This is a unique marker of transduction and is beneficial for a number of reasons. There is nothing that limits cell surface expression of CD34t, therefore its expression levels are analogous to the amount of TCR protein being made. CD34t can also be used to sort TCR transduced cells which allows for an easy method of attaining a pure TCR transduced T cell population. Following the CD34t, is a 3’ LTR sequence for genomic insertion. The SAMEN vector containing each WT or modified TCR described in the dissertation was used to generate high titer PG13 producer cell lines.

**First Round of DMF5 TCR Mutations**

The following seven modified TCRs were made using the WT DMF5 TCR sequence: αD26Y, βL98W, αD26Y/βL98W, αD26Y/αY50A/βL98W, αD26Y/αY50V/βL98W, αY50A, and αY50V. This was completed using a GENEART site
directed mutagenesis kit (Invitrogen). Mutagenesis was completed in the SAMEN vector with the primers listed below (IDT, Coralville, IA). Base changes are denoted by bold and underline.

αD26Y forward:
5’-CAACTGCACCTTACAGTTACACTGCACTTACAGTCCT-3’

αD26Y reverse:
5’-AGGACTGTAAGTGCACTGTGAACTGTAAGTGCACTTGG-3’

βL98W forward:
5’-TGTACTTCTGTGCCACGCTTGGTTCCTCGAAGCAGCTTTC-3’

βL98W reverse:
5’-GAAAGCTTCAGTTCCGAAGGACCAAACGTGGCAGAGGATGACA-3’

αY50A forward:
5’-CCTGAGTTGATAATGGTTCATACTCCATGGGTAAGAAAGAGATGG-3’

αY50A reverse:
5’-CCATCTTTCTTGTTCGCTGTGGAGGGCTATGAACATTACATCAAATCACTCAGGG -3’

αY50V forward:
5’-CCTGAGTTGATAATGGTTCATACTCCATGGGTAAGAAAGAGATGG-3’

αY50V reverse:
5’-CCATCTTTCTTGTTCGCTGTGGAGGGCTATGAACATTATCAAATCACTCAGGG -3’

Methylation and PCR amplification was performed using a C1000 Thermal Cycler (Bio-Rad, Hercules, CA) under the following conditions: 20 minutes at 37°C (one cycle), 2 minutes at 94°C (one cycle), 20 seconds at 94°C, 30 seconds at 57°C, and 5 minutes
at 68°C (18 cycles), and 5 minutes at 68°C (one cycle). Recombination reaction was performed at RT for 10 minutes.

Mutated vector DNA was transformed into *Escherichia coli* DH5α-T1R competent cells (Invitrogen) onto LB ampicillin plates (25 grams LB agar (Fisher, Hampton, NH) in 1 L de-ionized water supplemented with 100 µg/mL ampicillin (Sigma-Aldrich)) and colonies were expanded in superbroth (32 grams Tryptone (Fisher), 20 grams yeast extract (Fisher), 5 grams NaCl (Fisher), 1 liter de-ionized water) with 100 µg/mL ampicillin (Sigma-Aldrich)). Plasmid DNA from recombinant clones was isolated using a Miniprep kit (Qiagen). All mutagenesis products were sequenced (Genewiz, South Plainfield, NJ) to ensure no errors had occurred.

**Second Round of DMF5 TCR Mutations**

The following four DMF5 mutations were made using the αD26Y/βL98W DMF5 TCR sequence: αD26Y/αN52A/βL98W, αD26Y/βN52A/βL98W, αD26Y/αK68A/βL98W and αD26Y/βT57A/βL98W. This was completed using a GENEART site directed mutagenesis kit (Invitrogen). Mutagenesis was completed in the SAMEN vector with the primers listed below (IDT, Coralville, IA). Base changes are denoted by bold and underline.

αK68A forward:
5'- CAGCACAGCTCAATGCA GCCAGCCAGTATG -3'

αK68A reverse:
5'- CATACTGGCTGGCTGCA TGGATTGAGCTGTGCTG -3'

αN52A forward:
5'- GTTCATATACTCCGCT GTTGACAAAAAG-3'
αN52A reverse:
5'-CTTCTTTTCACCAGGGAGATATGAAC-3'
βT57A forward:
5'-TATTTCAATACTGCAGGTACCAGGGCAAAGGAGAAGTCCC-3'
βT57A reverse:
5'-GGGACTTCTCTTTGGCCGGGTACCTGCAGTATTTGAATA-3'
βN52A forward:
5'-CATCCATTATTCAGCTACTGCAGGTACC-3'
βN52A reverse:
5'-GGTACCTGCAGTACCTGGAATAATGGATG-3'

All mutagenesis and DNA isolation was performed with the same methods as described in the section above. All products were sequenced (Genewiz, South Plainfield, NJ) to ensure no errors had occurred.

HCV 1406 TCR Mutations

The following two modified TCRs were made using the WT HCV 1406 TCR sequence: αY59A and αY59V. This was completed using a GENEART site directed mutagenesis kit (Invitrogen). Mutagenesis was completed in the SAMEN vector with the primers listed below (IDT, Coralville, IA). Base changes are denoted by bold and underline.

αY59A forward: 5'-TATTATTTATTCTGGGGCAAGCAGCCTCCCAGC-3'
αY59A reverse: 5'-GCTGGGAGGCTGCTTGGCCAGAATAAATAATA-3'
αY59V forward: 5'-TATTATTTATTCTGGGTCAAGCAGCCTCCCAGC-3'
αY59V reverse: 5'-GCTGGGAGGCTGCTTGACCCAGAATAAATAATA-3'
All mutagenesis and DNA isolation was performed with the same methods as described in the section above. All products were sequenced (Genewiz, South Plainfield, NJ) to ensure no errors had occurred.

**Generating High Titer Producer Cell Lines**

Using a HEK293GP packaging cell line, retroviral supernatants were prepared and used to make a stable retroviral producer PG13 cell line expressing the TCRs in the SAMEN vector as described [280]. On day 0, 3 million HEK293GPs were plated in 10 cm poly-D-Lysine coated plates (Corning) in 10 mL complete medium and incubated overnight at 37°C in 5% CO₂. HEK293GP cells were transiently co-transfected with 20 µg retroviral SAMEN vector DNA and 5 µg of a plasmid containing the VSV envelope gene using 50 µL Lipofectamine 2000 (Invitrogen) on day 1. Transfection medium was replaced 6 hours later with 10 mL fresh complete medium and incubated for 48 hours at 37°C in 5% CO₂. On day 2, PG13 cells were seeded at 2 million in a 10 cm tissue culture plate in 10 mL complete medium and incubated at 37°C in 5% CO₂. On day 3, fresh HEK293GP viral supernatant was collected and filtered to sterilize using a 0.45 µm filter (Millex, Billerica, MA). PG13 media was replaced with 9 mLs of filtered HEK293GP viral supernatant and 3 mL complete medium. Plates were incubated for 72 hours at 37°C in 5% CO₂. On day 6, PG13 cells were collected and stained using anti-CD34-PE mAb (BioLegend) and analyzed for CD34 expression by flow cytometry. Four days later, cells were stained with an anti-CD34-PE (clone 561) mAb and CD34 positive cells were sorted for high and uniform expression using a BD FACSAria cell sorter (BD BioSciences, San Jose, CA) and the final PG13 cells were maintained in complete medium.
**T cell and Jurkat E6.1 Cell Transduction**

T cells derived from normal healthy donors were activated by adding 50 ng/mL anti-CD3 monoclonal antibody (Miltenyi Biotec, San Diego, CA), 300 IU/mL rhIL-2 (rhIL-2; Novartis Pharmaceuticals Cooperation, East Hanover, NJ), and 100 ng/mL rhIL-15 (NCI-Biological Resources Branch, Frederick, MD) to RPMI supplemented with 10% FBS on day 0. To make high titer retroviral supernatant, PG13 cell lines were seeded overnight at 8x10^6 cells/T-175 cm^2 cell culture flask at 37°C in 5% CO\textsubscript{2} on day 1. On day 2, 25 mLs of complete Iscove’s DMEM supplemented with 0.5 mLs (1 mM) sodium butyrate (Sigma-Aldrich) and 1 mL (10 mM) HEPES (Sigma-Aldrich) was added to flasks for 8-10 hours to stimulate virus production. Media was then replaced with fresh complete medium and incubated overnight at 37°C in 5% CO\textsubscript{2}. Fresh viral supernatants were collected on day 3 and filter sterilized to remove any cellular debris using 0.45 µm filters (Thermo Scientific, Waltham, MA).

Activated T cells were transduced by spinoculation on day 3 as described [330, 363, 365, 366]. Briefly, 24-well-flat-bottom-non-tissue-culture-treated plates were coated with 0.5mL/well 30 µg/mL Retronectin (Takara, Mountain View, CA) overnight. The next day, plates were blocked using 2% bovine serum albumin (BSA) in PBS (Thermo Scientific) for 30 minutes. Next, virus was loaded by adding 2 mL of fresh retroviral supernatant per well and plates were spun for 2 hours at 2,000xg at 32°C. 1 mL of 2x10^6 million/mL activated T cells in complete medium were added to the plates with 1 mL of fresh viral supernatant. The plates were spun again for 2 hours at 2,000xg at 32°C and then incubated overnight at 37°C in 5% CO\textsubscript{2}. After 24 hours, the TCR transduced T cells were transferred to cell culture flasks and plated at 1x10^6/mL in
complete medium. On day 7, transduction efficiency was determined by FACS analysis using anti-CD34-PE (clone 561) mAb. TCR transduced T cells were purified by positive selection using CD34 immunomagnetic beads (Miltenyi Biotec, San Diego, CA) and maintained in complete T cell medium at 37°C in 5% CO₂.

**Rapid Expansion Protocol**

Two days after CD34 enrichment, TCR transduced T cells were further expanded via a rapid expansion protocol (REP) to generate a large population of CD34⁺ TCR transduced T cells for T cell functional assays. 1x10⁶ T cells were cultured in a T-175 cm² cell culture flask with 200x10⁶ irradiated (50 Gy) allogeneic PBMCs (pooled from three normal donors) in 150 mL of complete T cell medium consisting of RPMI 1640 medium supplemented with 10% FBS, 300 IU/mL recombinant IL-2 (rhIL-2; Novartis Pharmaceuticals Cooperation, East Hanover, NJ), and 100 ng/mL recombinant human IL-15 (rhIL-15; Biologic Resources Branch, NCI, Frederick, MD), and 30 ng/mL anti-CD3 mAb (Miltenyi Biotech). T cells were incubated at 37°C in 5% CO₂ for 10 days and harvested for use in T cell functional assays.

**Immunofluorescence Staining**

PG13 cells were stained for CD34 surface expression by immunofluorescence using anti-CD34-PE (clone 581). This was done to confirm retroviral transductions were successful and to measure transduction efficiency. Briefly, cells were resuspended in 30 μL of 2% PBSA (PBS with bovine serum albumin) and incubated with 1 μL of mAb and incubated at RT in the dark for 20 minutes. Cells were washed with 2% PBSA and resuspended in 300 μL of 2% PBSA. T cell surface markers were stained by immunofluorescence using the following mAbs: anti-CD4-PE/Cy7 (clone RPA-T4), anti-
CD8-FITC (clone SK1), anti-CD3-APC/Cy7 (clone UCHT1), anti-CD34-AF700 (clone 581), and anti-CD107A-AmCyan (clone H4A3) (BioLegend). Briefly, cells were resuspended in 30 μL of 2% PBSA and incubated with 1 μL of mAb and incubated at RT in the dark for 20 minutes. Intracellular cytokines were stained by immunofluorescence using the following mAbs: anti-IL-2-PerCP/Cy5.5 (clone MQ1-17H12), anti-IFN-γ-PacBlue (clone 4S.B3), anti-IL-17A-Qdot 585 (clone BL168), anti-TNF-α-Qdot 705 (clone MAb11), anti-IL-4-APC (clone 8D4-8), and anti-IL-22-PE (clone 2G12A41). Briefly, cells were resuspended in 30 μL of 2% PBSA and incubated with 1.5 μL of mAb and incubated at RT in the dark for 20 minutes. T2 cells were stained by immunofluorescence using anti-HLA-A2-APC (clone BB7.2) mAb (BioLegend). Briefly, cells were resuspended in 30 μL of 2% PBSA and incubated with 1 μL of mAb and incubated at RT in the dark for 20 minutes. Tumor cells were stained by immunofluorescence using anti-CD9-FITC (clone HI9a) and anti-HLA-A2-APC mAb (BioLegend). Briefly, cells were resuspended in 30 μL of 2% PBSA and incubated with 1 μL of mAb and incubated at RT in the dark for 20 minutes. Cells were analyzed using an LSRFortessa flow cytometer (BD Biosciences, San Jose, CA) and the data was analyzed using FlowJo software (FlowJo Enterprise, Ashland, OR).

**Cytokine Release Assay**

Antigen reactivity of the TCR transduced T cells or Jurkat E6.1 cells was measured in cytokine release assays as described [94, 271, 366]. Briefly, T2 stimulators were loaded with 10 μg/mL of peptide two hours prior to coculture and incubated at 37°C in 5% CO₂. 1x10⁵ washed and re-suspended responder T cells and 1x10⁵ washed and re-suspended stimulator cells were cocultured in a 1:1 ratio in 96-well U-bottom
tissue culture plates (Corning) in 200 µL complete medium. Phorbol 12-Myristate 13-Acetate (PMA) (Thermo Fisher) was added to cocultures using Jurkat E6.1 cells at 10 ng/mL. Cocultures were incubated at 37°C in 5% CO₂ for 18-20 hours. Plates were centrifuged at 1,500 RPM and supernatants were collected for analysis of cytokine release.

The amount of cytokine released was measured via sandwich ELISA using monoclonal antibodies to IFN-γ or IL-2 (BioLegend, San Diego, CA). Briefly, 96-well plates were coated overnight at 4°C with human IFN-γ capture mAb or IL-2 capture mAb. The next day, plates were washed with ELISA wash buffer (1X PBS, 0.01% Tween) then blocked with 200 µL 1X Assay Diluent (phosphate buffered saline solution containing bovine serum) for 1 hour at RT on a shaker. Plates were washed again with ELISA wash buffer and 100 µL of samples and standards were added. After 2 hours of shaking at RT, plates were washed and 100 µL of the enzyme-conjugated IFN-γ or IL-2 detection mAb was added. After 1 hour of shaking at RT, 100 µL of Avidin-HRP was added and incubated on the shaker for 30 minutes at RT. Plates were then washed and 100 µL of TMB Substrate Solution was added. Plates were incubated at RT in the dark for 20 minutes or until color developed in standard curve. 100 µL of 2N H₂SO₄ was used to stop the reaction. The absorbance of the plates was read at 450 nm using a spectrophotometer.

**Polyfunctional T cell Assay**

Polyfunctional antigen reactivity of TCR transduced T cells was measured by CD107A and intracellular cytokine expression as described [95]. 3x10⁵ TCR transduced T cells were cocultured with 3x10⁵ peptide loaded T2 cells in 96-well U-bottom tissue
culture plates in 200 μL of RPMI supplemented with 10% FBS at 37°C, 5% CO₂ for 5 hours. 5.0 ng/mL brefeldin-A, 2.0 nM monensin (BioLegend), and anti-CD107A-AmCyan mAb were added at the beginning of coculture. After 5 hours, cells were stained for 20 minutes for the following surface markers: anti-CD4-PE/Cy7, anti-CD8-FITC, anti-CD3-APC/Cy7, and anti-CD34-AF700 (BioLegend). Cells were fixed, permeabilized, and stained for the following intracellular markers: anti-IL-2-PerCP/Cy5.5, anti-IFN-γ-PacBlue, anti-IL-17A-Qdot 585, anti-TNF-α-Qdot 705, anti-IL-4-APC, and anti-IL-22-PE (BioLegend). Data were acquired using an LSRFortessa flow cytometer. Staining profiles were gated and analyzed using FlowJo.

**Polymonal Gating and Analysis**

Lymphocyte populations were determined by FSC (forward scatter) vs. SSC (side scatter). TCR transduced T cell populations were determined by CD34⁺CD3⁺ gating. TCR transduced T cells were further gated on CD4 CD8⁺ and CD4⁺CD8⁻ populations and subsequent single functional markers. An example of gating on cell surface markers and functional markers is shown in Figures 4-6. Boolean gating was performed in FlowJo for CD107A, IFN-γ, TNF-α, IL-17A, IL-2, IL-4, and IL-22 to give 2⁷ potential functional phenotypes. After sequential gating in FlowJo, Pestle (NIH, Bethesda, MD) formatted the multivariate datasets and performed background subtraction using the irrelevant peptide (T2 + HCV) for each respective DMF5 TCR to account for background in immunofluorescence staining [367]. SPICE (Simplified Presentation of Incredibly Complex Evaluations) (NIH, Bethesda, MD) was utilized for its ability to compare the distributions of all the polyfunctional parameters [367].
Figure 4. Gating Strategy for Cell Surface T cell Markers. Samples were first gated on side scatter (SSC) and forward scatter (FSC) to isolate lymphocyte population. Cells in the lymphocyte gate were then gated on CD3 and CD34, double positive population indicates TCR transduced T cells. TCR transduced T cells were subsequently gated on CD4 and CD8.

Figure 5. Gating Strategy for Functional T cell Markers. CD3+CD34+ and either CD4+CD8- or CD4-CD8+ (depicted above) cells were subsequently gated on the following individual functional markers: IFN-γ, CD107A, TNF-α, IL-2, IL-17A, IL-4, and IL-22. One representative donor and experiment is shown with T cells expressing the WT DMF5 TCR. (a) Gating strategy against T2 cells loaded with irrelevant, HCV peptide. (b) Application of gating strategy against T2 cells loaded with the MART-1 9mer peptide.
Figure 6. Gating Strategy for Functional T cell Markers. Application of previous gating strategy on CD4-CD8+ T cells transduced to express the αD26Y DMF5 TCR. One representative donor and experiment is shown. (a) Application of gating strategy against T2 cells loaded with irrelevant, HCV peptide. (b) Application of gating strategy against T2 cells loaded with the MART-1 9mer peptide.

Background subtraction can result in values below zero and values between 0.001% - 0.09%. One of the advantages of SPICE is that it has a threshold approach. Therefore, cool plots were generated in SPICE to visualize any positive functional phenotype present over 0.1%.

LDH (lactate dehydrogenase) Assay

Lysis of tumor targets was measured using a Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific). 1x10⁵ washed and re-suspended TCR transduced T cells and 1x10⁵ washed and re-suspended target cells were cocultured in a 1:1 ratio in triplicate in 96-well U-bottom tissue culture plates in 100 µL medium. Cells for the following controls were also plated: effector cell spontaneous LDH release, target cell spontaneous LDH release, target cell maximum LDH, volume correction, and culture medium background. Cocultures were incubated at 37°C in 5% CO₂ for 18 hours. 45 minutes before harvesting the supernatant, 10 µL of provided Lysis Buffer was added to wells.
containing the target cell maximum LDH control to determine the maximum lysis and to wells containing the volume correction control to account for volume increase caused by the addition of the Lysis Buffer. Plates were spun at 300 x g for 3 minutes and 50 μL of supernatant was collected and transferred to 96-well flat-bottom tissue culture plates and mixed with 50 μL of provided Reaction Mixture for 30 minutes at RT, in the dark. Reaction was stopped with 50 μL of provided Stopping Solution. Absorbance was read at 490 nm and 680 nm using a spectrophometer. Absorbance value at 680 nm (background signal from spectrophometer) was subtracted from the absorbance value at 490 nm to determine LDH activity. The percent cytotoxicity was calculated using the following formula: % cytotoxicity = ((experimental value – effector cell spontaneous control – target cell spontaneous control) / (target cell maximum control – target cell spontaneous control)) x 100.

**Combinatorial Peptide Library**

Functional assays using a combinatorial peptide library revealed results pertinent to the conclusions of these studies. These specific experiments were completed by the Baker Lab at the University of Notre Dame. These results were obtained as follows: A decameric combinatorial peptide library was obtained from Pepscan (Lelystad, Netherlands). The library excluded cysteine and fixed p2 and p10 to leucine and valine, respectively, for a total of $19^8$ (approximately $1.7 \times 10^{10}$) peptides. The library was composed of 152 sub-libraries in which each position of the peptide, except p2 and p10, was fixed at each amino acid, excluding cysteine. The library scan was conducted as previously described [368, 369]. Briefly, $1 \times 10^5$ T2 cells were loaded with 100 μM total peptide concentration of each sub-library for two hours at 37 °C. An equal number of
PMA simulated (50 ng/mL) Jurkat 76 CD8+ cells transduced with the WT DMF5 TCR, αD26Y/βL98W DMF5 TCR, or αD26Y/αY50A/βL98W DMF5 TCR were added and cocultured for 18-20 hours at 37°C, after which supernatant was harvested and assayed for IL-2 via ELISA. Combinatorial peptide library scans were repeated three times with freshly generated cells and the results were averaged.

**Crystallization and Structural Analysis**

TCR/pMHC crystal structures are shown in this dissertation and are a critical component of the data analysis. These experiments were completed by the Baker Lab at the University of Notre Dame. The crystal structures were obtained as follows: Crystals of the αD26Y/βL98W DMF5 TCR/MART-1 decamer/HLA-A2 complexes were grown from 12% PEG 3350, 0.25 M MgCl₂ buffered with 0.1 M HEPES (pH 8.0) at 25°C. Crystallization was performed using sitting drop/vapor diffusion. For cryoprotection, crystals were transferred into 20% glycerol/80% mother liquor for 30 seconds and immediately frozen in liquid nitrogen. Diffraction data were collected at the 22ID (SER-CAT) beamlines at the Advanced Photon Source, Argonne National Laboratories, Argonne, IL. Data reduction was performed with HKL2000. The ternary complexes were solved by molecular replacement using PHENIX and Protein Data Bank (PDB) entry 3QDG as the reference model [370]. Rigid body refinement, followed by translation/libration/screw (TLS) refinement and multiple steps of restrained refinement were performed. TLS groups were automatically chosen by phenix.refine. Once defined, TLS parameters were included in all subsequent steps of the refinement. Anisotropic and bulk solvent corrections were taken into account throughout refinement. After TLS refinement, it was possible to unambiguously trace the position of peptides
and TCR CDR loops in all structures against $\sigma_A$-weighted $2F_o-F_c$ maps. Evaluation of models and fitting to maps were performed using COOT [371]. The template structure check in WHATIF [372] and MolProbity [373] was used to evaluate the structures during and after refinement. Atomic positioning was verified with an iterative-build OMIT map calculated in PHENIX [374]. Structures were visualized using PyMOL. Analysis of hydrogen bonds was performed with HBPlus [375], using hydrogen-acceptor maximum distance of 2.7 Å and a donor-acceptor maximum distance of 3.6 Å. Solvent accessible surface areas were measured in Discovery Studio (Accelrys Inc.) using a probe radius of 1.4 Å. The structure has been deposited with the Protein Data Bank (PDB ID 4L3E).

**Surface Plasmon Resonance**

3D affinity measurements are shown in this dissertation and are a critical component of the data analysis. These experiments were completed by the Baker Lab at the University of Notre Dame. The 3D affinity measurements were obtained as follows: A Biacore T200 instrument was used to perform surface plasmon resonance experiments. Amine coupling was used to immobilize the TCR to CM-5 sensor chips at 1500-2000 response units and pMHC complex was injected as analyte in all experiments. All samples were thoroughly dialyzed in HBS-EP buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P-20) followed by degassing for at least 15 minutes before use. pMHC injections covered a concentration range of 0.5-150 μM at a flow rate of 5 μL/minute at 25°C. Multiple steady-state data sets were globally fit using a 1:1 Langmuir binding model with BIAevaluation 4.1 to extend the range and accuracy of measurements as previously described [167, 376]. Measurements of TCR binding to the MMW/HLA-A2 complex were performed using a
kinetic titration assay at 25°C [377], with chip densities near 150 RU for the 
αD26Y/βL98W TCR and 500 RU for the αD26Y/αY50A/βL98W TCR. pMHC 
concentrations ranged from 32-500 nM for the αD26Y/βL98W TCR and 1-16 μM for the 
αD26Y/αY50A/βL98W TCR. Kinetic titrations used a flow rate of 100 μL/minute.

**Micropipette Adhesion Frequency Assay**

2D affinity measurements are shown in this dissertation and are a critical 
component of the data analysis. These experiments were completed by the Evavold 
Lab at the University of Utah. The 2D affinity measurements were obtained as follows:
The relative 2D affinity of JurkatE6.1 cells TCR transduced to express the WT and 
mutant DMF5 TCRs was measured using the previously characterized 2D micropipette 
adhesion frequency assay [162, 378-380]. Briefly, RBCs coated with a range of Biotin-
LC-NHS (BioVision, Milpitas, CA) were then coated with 0.5 mg/mL streptavidin 
(Thermo Scientific) followed by 1 μg of biotinylated MART-1 10mer monomer. The 
adhesion frequency between TCR transduced JurkatE6.1 cells and ligand coated RBCs 
aspirated on opposing pipettes were observed using an inverted microscope. An 
electronically controlled piezoelectric actuator repeated JurkatE6.1 cell contact with the 
pMHC coated RBCs 50 times for controlled contact area (A_c) and time (t). Upon 
retraction of the T cell, adhesion (binding of TCR-pMHC) was observed by distention of 
the RBC membrane, allowing for quantification of adhesion frequency (P_a) at 
equilibrium. Surface pMHC (m_i) and TCR beta (m_r) densities were determined by flow 
cytometry using MHC class I anti-human HLA-A2 –PE (clone BB7.2) mAb (BioLegend) 
and anti-human α/β TCR-PE (clone IP26) mAb (BioLegend), both at saturating 
concentrations, and BD QuantiBRITE PE Beads for standardization (BD Biosciences,
San Jose, CA). The calculation of molecules per area were done by dividing the number of TCR and pMHC per cell by the respective surface areas. The relative 2D affinities were calculated using the following equation: $A_C K_a = -\ln [1 - P_a(1)]/m_r m_l$. Normalized adhesion frequency was calculated using the equation $(-\ln(1 - P_a(s))/mpMHC)$. Geometric mean of affinities and normalized adhesion bonds are reported ± SEM.

**In vivo NSG A2 Tumor Growth Models**

The *in vivo* studies completed in this dissertation utilized an established human xenograft mouse model. Using immunocompromised mice, xenografts are advantageous to examine therapeutic efficacy against human tumors [381-383]. NOD.Cg-Prkdc<sup>scid</sup> I2rg<sup>tm1Wjl</sup>/SzJ (NSG) HLA-A2 immunodeficient mice were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were housed in our AALAC-approved animal facility at the Loyola University Health Science Campus. The hind flanks of mice were shaved at least one day prior to tumor challenge. Under inhalatory anesthesia, 6-8 week old NSG A2 mice were inoculated subcutaneously with $5 \times 10^6$ MEL 624 tumor cells in 100 μL PBS. Tumor growth was assessed 2-3 times a week by caliper measurements. Tumor areas were determined by the following formula: area = $(3.14 \times \text{length} \times \text{width})/4$. All TCR transduced T cells used for therapeutic injection were CD34 enriched using immunomagnetic beads (Miltenyi Biotec). All TCR transduced T cell populations completed a REP (previously described) for 10-12 days to obtain a large number of TCR transduced T cells for therapy. High and uniform CD34 expression was confirmed amongst treatment groups prior to injection. The number of injected TCR transduced T cells is stated in subsequent sections in each respective experimental
model. Mice were sacrificed when tumors reached 150 mm² in size or 10% of body weight by CO₂ inhalation followed by cervical dislocation.

**Human PBMC Engraftment Prior to Tumor Challenge**

NSG mice have been previously engrafted with human hematopoietic stems cells to recapitulate a human immune system in the mouse [384, 385]. We developed a model of engraftment using human PBMC to determine if engraftment supported introduced human TCR transduced T cells. First, human PBMCs were thawed and cultured in RPMI supplemented with 10% FBS, 300 IU/mL rhIL-2, and 100 ng/mL rhIL-15 and were incubated at 37°C in 5% CO₂ for 24 hours. After 24 hours, 6-8 week old NSG HLA-A2 mice were injected with 10x10⁶ human PBMCs in 100 μL PBS either via retro-orbital or intraperitoneal route of injection. The persistence of engrafted cells in the blood was monitored weekly. Briefly, a 3mm Goldenrod lancet (Fisher Scientific) was used to obtain one drop of blood from the facial vein. Red blood cells were lysed by incubation with 200 μL of ACK Lysis Buffer (Lonza, Basel, Switzerland) for 5 minutes at RT. Cells were washed twice with 1 mL of 2% PBSA and were immunofluorescently labeled using human anti-CD3-APC/Cy7 antibody (BioLegend). 7 days after PBMC engraftment, NSG A2 mice were inoculated subcutaneously with 5x10⁶ MEL 624 tumor cells in 100 μL PBS. Tumor growth was assessed 2-3 times a week by caliper measurements. 17 days post tumor challenge, or when tumors reached about 4 x 4 mm, 10x10⁶ human TCR transduced T cells in 100 μL PBS were injected retro-orbitally. The persistence of TCR transduced T cells was monitored weekly in the blood of mice as described above. Cells were immunofluorescently labeled using human anti-CD3-APC/Cy7 and human anti-CD34-PE antibodies (BioLegend). Mice were closely
observed for cachexia as a potential indicator of autoimmunity. Mice were sacrificed when tumors reached 150 mm$^2$ in size or 10% of body weight by CO$_2$ inhalation followed by cervical dislocation.

**Cytokine Support of Introduced Human TCR Transduced T cells**

Cytokines were administered to NSG A2 mice to determine their effect on introduced human TCR transduced T cell persistence. 6-8 week old NSG A2 mice were inoculated subcutaneously with 5×10$^6$ MEL 624 tumor cells in 100 µL PBS. Tumor growth was assessed 2-3 times a week by caliper measurements. 17 days later, or when tumors reached about 4 x 4 mm, 10x10$^6$ TCR transduced human T cells in 100 µL PBS were injected retro-orbitally. Beginning on day 17, groups of mice were given 2.5 µg rhIL-15 in 100 µL PBS every 3 days or 60,000 IU rhIL-2 in 100 µL PBS twice a day for four days, then once a day every 3 days via intraperitoneal injection [386, 387]. Tumors from one to two mice per group were processed on day 16 using a tumor dissociation kit (Miltenyi Biotec) to examine the persistence of TCR transduced T cells. Spleens from one to two mice per group were processed on day 16 to examine the persistence of TCR transduced T cells. Briefly, spleens were manually disrupted using the plunger of a 3 mL syringe over a 70 µm cell strainer (Corning). Red blood cells were lysed by incubation with 200 µL of ACK Lysis Buffer (Lonza, Basel, Switzerland) for 5 minutes at RT. Tumor and spleen derived cells were washed twice with 1 mL of 2% PBSA and were immunofluorescently labeled using human anti-CD3-APC/Cy7 and human anti-CD34-PE antibodies (BioLegend). Mice were sacrificed when tumors reached 150 mm$^2$ in size or 10% of body weight by CO$_2$ inhalation followed by cervical dislocation.
Checkpoint Blockade with Human TCR Transduced T cells

Checkpoint blockade was administered to NSG A2 mice to determine the effect on introduced human TCR transduced T cell persistence. 6-8 week old NSG A2 mice were inoculated subcutaneously with $5 \times 10^6$ MEL 624 tumor cells in 100 μL PBS. Tumor growth was assessed 2-3 times a week by caliper measurements. 17 days later, or when tumors reached about 4 x 4 mm, $10 \times 10^6$ TCR transduced human T cells in 100 μL PBS were injected retro-orbitally. Beginning on day 17, groups of mice were given 0.25 mg of anti-PD-1 mAb (Bio X Cell, West Lebanon, NH) in 100 μL Buffer (Bio X Cell, West Lebanon, NH) every 3 days (10 mg/kg mouse) via intraperitoneal injection. Mice were sacrificed when tumors reached 150 mm$^2$ in size or 10% of body weight by CO$_2$ inhalation followed by cervical dislocation.

*In vivo* CTL Assay

An *in vivo* CTL assay was performed to determine if TCR transduced T cells could elicit target specific killing *in vivo*. 6-8 week old NSG A2 mice were injected with 100 μL PBS or $10 \times 10^6$ WT DMF5 TCR transduced T cells in 100 μL PBS on day 0. On day 1, HLA-A2$^+$ PBMCs were pulsed with the MART-1 9mer peptide for 2 hours, as previously described. HLA-A2$^+$ PBMCs were incubated with 0.5 μM of carboxyfluorescein succinimidyl ester (CFSE) (Thermo Fisher) for 8 minutes at 37˚C. MART-1 9mer pulsed HLA-A2$^+$ PBMCs were incubated with 5 μM of CFSE (Thermo Fisher) for 8 minutes at 37˚C. CFSE labeled cells were washed 3 times in 25 mLs of warmed RPMI supplemented with 10% FBS. CFSE high and CFSE low cells were mixed in a 1:1 ratio. $12 \times 10^6$ CFSE labeled cells in 100 μL PBS were injected into mice via retro-orbital route. Non-transferred CFSE labeled cells were kept *in vitro* at 37˚C as
the non-transferred control. On day 3, spleens were isolated and processed as described above. Cells were labeled with anti-HLA-A2-APC mAb (BioLegend) and analyzed via flow cytometry. Ratio = CFSE low:CFSE high. The % MART-1 specific lysis was determined by the following formula: % specific lysis = ((1-(non-transferred control ratio))/experimental ratio) x 100.

**Statistical Analysis**

Comparisons of WT DMF5 TCR vs. mutant DMF5 TCRs were evaluated by two-way ANOVA using Sidak’s multiple comparisons test. **** P <0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05. Results are represented as mean ± standard error of the mean (SEM) unless otherwise indicated. Experiments were repeated 2-3 times in 2-4 independent donors.
CHAPTER THREE

RESULTS

Structure-Guided Approach and DMF5 Mutations

Despite the success observed in clinical trials where TCR gene-modified T cells were used, there still remains areas in which improvement is needed. These areas include both the efficacy and the safety of the treatment in patients. One of the drawbacks of using TCRs that target self-antigens in TCR gene-modified T cells is that they generally harbor a low affinity, due to negative selection during T cell development. Negative selection is critical in order to eliminate potentially autoreactive T cells [41, 388]. Therefore, their anti-tumor efficacy can be suboptimal for treatment. One strategy to enhance anti-tumor efficacy is to enhance the affinity of the TCR. Yeast or phage display strategies have been implemented for affinity enhancement [295, 296, 343, 344, 389]. Albeit, affinity enhanced TCRs can result in unanticipated cross-reactivity in T cells and have resulted in deaths in clinical trials [5, 6]. Not all affinity enhanced TCRs have elicited adverse events [302]. However, when modifying a TCR, more meticulous strategies are needed in order to fine-tune the specificities of a TCR.

Here, in collaboration with the Baker’s laboratory, we have developed a novel structure-guided design approach to fine-tune TCR antigen specificity while simultaneously reducing cross-reactivity. This strategy is based on the principles of positive and negative design. Positive design was proposed to introduce favorable
interactions between the TCR and the peptide. Conversely, negative design was proposed to weaken existing favorable interactions between the TCR and the MHC. Herein, we used the DMF5 TCR and the MART-1 peptide in the context of HLA-A2 for a model to test this structure-guided approach. A graphical depiction of this positive and negative design strategy is shown in Figure 7. Both the MART-1 nonameric (positions 27-35) and decameric (positions 26-35) epitopes are recognized by MART-1 reactive T cells [314, 324-326, 390]. It was further demonstrated that the decameric epitope bound more tightly to the HLA-A2 molecule than the nonameric epitope [391]. Mutation of the decameric anchor residue, alanine, to a leucine, further enhanced immunogenicity and binding to the HLA-A2 molecule, more so than anchor modification with the nonameric epitope [391, 392]. The DMF5 TCR recognized both the MART-1 nonameric epitope (AAGIGILTV) and the anchor-modified decameric epitope (ELAGI GILTV) in the context of HLA-A2. In these studies, MART-1 9mer will refer to the AAGIGILTV peptide, and MART-1 10mer will refer to the anchor modified, ELAGIGILTV peptide. The crystal structures of the DMF5 TCR/pMHC indicated that despite differences in the MART-1 9mer and 10mer peptide conformations in HLA-A2, the DMF5 TCR engaged both of the peptides with identical binding modes. The superimposed structures of the DMF5 TCR bound to the MART-1 9mer and 10mer peptides/HLA-A2 are shown in Figure 8 [325]. Specifically, the conformations of the CDR loops are the same between the two peptides in both the side and top view. For these reasons, the crystal structure of the DMF5 TCR engaged with HLA-A2 and the MART-1 10mer was utilized for structure-based design due to the MART-1 10mer’s enhanced binding and stability in the HLA-A2 molecule [351].
In collaboration with the Baker lab, we have developed a positive and negative structure-guided design approach to enhance antigen specificity and reduce cross-reactivity. This was first implemented in a model using the DMF5 TCR and MART-1 peptide. The MART-1 peptide in the context of the HLA-A2 MHC class I molecule is shown in purple and green, respectively. The DMF5 TCR is shown in blue and CD3 is shown in red. The left complex depicts the WT DMF5 TCR expressed on a T cell, engaging with the MART-1/HLA-A2 complex on a melanoma cell. The right TCR depicts the combinatorial strategy of positive and negative (+/-) design in the DMF5 TCR. The TCR is expressed on a T cell and engages with the MART-1/HLA-A2 complex on a melanoma cell. However, positive mutations, depicted by the red/orange bursts, were introduced in the DMF5 TCR and are proposed to enhance MART-1 peptide specificity. Conversely, negative mutations, depicted by the white space between the TCR and HLA-A2, were introduced in the DMF5 TCR and are proposed to weaken TCR binding with the HLA-A2 MHC class I.
Figure 8. Crystal Structure of the DMF5 TCR and MART-1 Nonamer and Decamer Peptide/HLA-A2 Complexes. The DMF5 TCR binding with the nonamer peptide/HLA-A2 complex is depicted in yellow (AAGIGILTV). The DMF5 TCR binding with the decameric/HLA-A2 complex is depicted in purple (ELAGIGILTV). (a) Side view of the DMF5 TCR complex with the nonamer and decamer peptide/HLA-A2 complexes. (b) Top view of superimposition from panel a. Identical overlap of CDR loops of the DMF5 TCR over the two peptide/HLA-A2 complexes is shown. These structures were provided by the Baker lab [370].

The structure-guided design strategy, developed in collaboration with the Baker lab, was first implemented in the DMF5 TCR. Two positive mutations and two negative mutations were designed based on the crystal structure of the DMF5 TCR/MART-1 10mer/HLA-A2 tri-molecular complex. DMF5 TCR residues were identified for positive mutations by simulating different point mutations in the TCR within 5.5 Å of the pMHC and were chosen based on their predicted ability to enhance the affinity of the DMF5 TCR [351, 393, 394]. The 5.5 Å distance threshold was set in order to avoid TCR
mutations enhancing binding with the MHC. The first positive mutation substituted an aspartic acid with a tyrosine at position 26 (αD26Y) in the CDR1 region of the TCR alpha chain. This mutation was designed to enhance charge complementarity with the N-terminal region of the MART-1 peptide. The X-ray structure of the WT DMF5 TCR compared to the αD26Y DMF5 TCR is shown in Figure 9 [351].

More specifically, in the complex with the WT TCR, αD26 and the glutamic acid in position 1 of the MART-1 10mer peptide are in close proximity. Consequently, the negative charges between the aspartic acid and the glutamic acid result in an unfavorable repulsion of charge (Figure 10) [349]. Furthermore, the αD26 side chain also induces charge repulsion with the side chain of the glutamic acid at position 58 in the HLA-A2 α1 helix [349]. Tyrosines are large residues and thus can participate in a number of van der Waals forces and electrostatic interactions. The rigid, bulky, and amphipathic nature of tyrosine provides structural utility [395]. This explains why mutations that have been shown to enhance affinity frequently replaced small polar or charged amino acids with large hydrophobic or amphipathic amino acids [72]. The αD26Y TCR mutation eliminates the charge repulsion with the peptide and consequently enhances charge complementarity with the peptide and increases the TCR affinity to pMHC (MART-1/HLA-A2). The peptide is only a small exposed area compared to the MHC and the peptide and MHC are tightly packed together. This makes it quite difficult, even when utilizing a meticulous targeted design strategy, to identify residues and mutations in the TCR that will only affect TCR binding with peptide and not the MHC as well.
Figure 9. X-ray Structure of WT and αD26Y DMF5 TCRs in Complex with pMHC. The DMF5 TCR alpha chain is yellow, the HLA-A2 is green, and MART-1 peptide is magenta. (a) The WT residue, αD26, shown in purple. (b) The mutated, αY26, shown in purple. Hydrogen bonds involved in the side chain and bound water molecule are depicted by dashed lines. These structures were provided by the Baker lab [351].

Figure 10. Structural Impact of the αD26Y DMF5 TCR Mutation on Charge Repulsions with the pMHC. The HLA-A2 complex is shown in blue, the MART-1 10mer peptide is shown in yellow, and the DMF5 TCR is shown in pale brown. (a) In the WT DMF5 TCR, the αD26 exhibits unfavorable charge repulsion with the glutamic acid at position 1 of the MART-1 10mer peptide and with the glutamic acid at position 58 in the HLA-A2 complex. (b) In the αD26Y DMF5 TCR, the charge repulsion with the peptide and MHC is eliminated. These structures were provided by the Baker lab [349].
A schematic of how the αD26Y TCR mutation alters unfavorable charge repulsion to favorable charge repulsion with the pMHC is shown in Figure 10 [349]. In conclusion, the αD26Y TCR mutation is a positively designed mutation proposed to enhance binding with the MART-1 peptide by enhancing charge complementarity with the N-terminal region of the MART-1 peptide.

The αD26Y TCR mutation was proposed to enhance charge complementarity with the N-terminal region of the MART-1 peptide. However, other epitopes that conform to similar structures in their N-terminal region could consequently be recognized with this mutation. Therefore, a different positive mutation, βL98W, was designed in the CDR3 region of the DMF5 TCR beta chain, to be more MART-1 specific [351]. Because the CDR3 loops of TCRs are most often positioned over the center of the peptide to make contact with the peptide, the βL98W TCR mutation was designed to target a specific residue in the MART-1 peptide [71]. This TCR mutation was proposed to improve shape complementarity with the leucine at position 7 or 8 in the 9mer or 10mer MART-1 peptide, respectively. The X-ray structure of the WT DMF5 TCR compared to the βL98W DMF5 TCR is shown in Figure 11 [351]. The βL98W TCR mutation was chosen based upon its ability to enhance predicted TCR affinity against the MART-1/HLA-A2 complexes [351]. As mentioned previously about tyrosine, tryptophan is also a bulky and amphipathic amino acid and thus, provides structural and chemical value, and enhanced the DMF5 TCR affinity against the MART-1/HLA-A2 complex [72, 395]. Furthermore, in a previously generated peptide panel comprised of 9mer epitopes selected upon their sequence homology with the MART-1 peptide, the leucine in position 7 was absent in about 60% of the sequence homologous epitopes [327].
Figure 11. X-ray Structure of WT and βL98W DMF5 TCRs in Complex with pMHC. The DMF5 TCR beta chain is pale pink, the HLA-A2 is green, and MART-1 peptide is magenta. (a) The WT residue, βL98, shown in purple. (b) The mutated, βW98, shown in purple. Structures were provided by the Baker lab [351].

This suggested the βL98W TCR mutation might have less of an impact on the majority of MART-1 mimics in terms of enhancing binding and recognition. Overall, the βL98W DMF5 TCR mutation is the second positively designed TCR mutation, and is proposed to improve shape complementarity specifically with the leucine at position 7 or 8 in the 9mer or 10mer MART-1 peptide, respectively.

The field generally considers high affinity TCRs optimal for T cell function and therapeutic efficacy [180, 396]. Therefore, we wanted to determine the effect of binding and antigen specificity with a high affinity DMF5 TCR variant by combining the two previously described TCR mutations. More specifically, we wanted to determine how the combination of the two positive DMF5 TCR mutations (αD26Y and βL98W) in the αD26Y/βL98W double mutant DMF5 TCR, altered the recognition of MART-1 compared to either single mutant DMF5 TCRs. This combination was proposed to result in a
DMF5 TCR variant with substantial enhancement towards the MART-1/HLA-A2 complex due to the additive binding enhancements. The X-ray crystal structure of the WT DMF5 TCR overlaid with the αD26Y/βL98W DMF5 TCR is shown in Figure 12 [351]. Compared to the structure bearing the WT DMF5 TCR, there were no alterations of the interface TCR CDR loops or the peptide with the αD26Y/βL98W TCR, indicating this structure-based design strategy, utilized to identify specific residues for mutation, did not disrupt the TCR/pMHC interface or the neighboring side chains. Furthermore, compared to the WT TCR, the tyrosine and tryptophan mutant side chains more expansively make direct contact with the MART-1 peptide in the αD26Y/βL98W TCR [351]. In summary, the αD26Y/βL98W DMF5 TCR is proposed to enhance MART-1 peptide binding more than either single mutant DMF5 TCRs and this is depicted in the crystal structures.

This structure-guided approach is based upon the idea of positive and negative design. Even though the αD26Y/βL98W DMF5 TCR did not alter the CDR loops compared to the WT TCR in the TCR/pMHC complex, it is possible that these mutations could enhance off-target binding with MART-1 “like” epitopes. Therefore, introduction of negative mutation would offset this potentially enhanced off-target cross-reactivity. This is of critical importance because sole implementation of positive mutations, or sole introduction of only favorable interactions with the peptide could allow for unwanted effects on specificity. Thus, negative mutations were implemented to offset or compliment the DMF5 TCR mutations made to enhance peptide binding. Negative mutations were generated by identifying a TCR residue that if mutated, would weaken TCR binding with the MHC.
Figure 12. X-ray Structure of the WT TCR Overlaid with the αD26Y/βL98W DMF5 TCR in Complex with pMHC. The DMF5 TCR alpha chain is yellow, the DMF5 TCR beta chain is pale pink, the HLA-A2 is green, and MART-1 10mer peptide is magenta. The α26 TCR residue is shown in purple (above N-terminal end of MART-1 peptide). Both the aspartic acid and tyrosine residues are depicted. The β98 TCR residue is shown in purple (closer to C-terminal end of MART-1 peptide). Both the leucine and tryptophan residues are depicted. Structure was provided by the Baker lab [351].

The αY50 residue in the CDR2 region of the DMF5 TCR alpha chain was identified and chosen for designing negative mutations for multiple reasons. First, αY50 makes contact with an evolutionarily conserved and exposed region of HLA-A2. Specifically, αY50 makes contact with the glutamic acid at position 154, the glutamine at position 155, and the alanine at position 158 in the α2 helix of HLA-A2 [350]. Studies indicated that up to 16% of sequenced TCRs have a tyrosine at this residue, however, other amino acids at this residue still demonstrate conserved interaction with the same
area of HLA-A2 [37]. For example, in all known structures of Vα12-2 TCRs bound to pMHC, the tyrosine at position 50 interacts with the same residues in the HLA-A2 α2 helix [397]. It has been demonstrated that this TCR residue is important for interaction with MHC class I in many other TCRs as well [398]. Furthermore, evidence suggest that tyrosines are one of the more frequently used amino acids in prominent, conserved interactions. The large amphipathic nature of tyrosine allows it to maintain van der Waals forces more due to its size, rather than geometry. Overall, this tyrosine at position 50 in the DMF5 TCR alpha chain could be important for TCR binding with MHC because it can accommodate variation in structural changes within the TCR upon binding different peptides in the context of MHC [337].

We and the Baker laboratory believed the αY50 DMF5 TCR residue was a suitable residue for designing negative mutations, for the reasons described above. Two different TCR mutations were made at this residue: αY50A and αY50V. The tyrosine to alanine mutation was proposed to lose all contact with the HLA-A2 at positions 154, 155, and 158, whereas the tyrosine to valine mutation was proposed to weaken binding with HLA-A2 at positions 154, 155, and 158, but not lose all contact. The X-ray structure of the αY50A and αY50V DMF5 TCR mutations compared to the WT DMF5 TCR is shown in Figure 13. Overall, the crystal structures indicated no structural perturbations in the TCR with both of these mutations, aside from the directed removal or weakening of bonds with the α2 helix of HLA-A2.
Figure 13. X-ray Structure of αY50A and αY50V DMF5 TCR Mutations in Complex with pMHC. The DMF5 TCR alpha chain is yellow, the HLA-A2 is green, the WT αY50 residue is purple, and MART-1 peptide is magenta. (a) The αA50 is shown in orange. (b) The αV50 is shown in red. Structures were provided by the Baker lab.

The idea for utilizing this design strategy was not necessarily just to enhance DMF5 TCR affinity with the MART-1/HLA-A2 complex, but rather the idea was to redistribute the binding free energy to be more focused on the MART-1 peptide and less focused on the MHC. The combination approach (positive and negative design) is imperative in order to fine tune the antigen specificity of the DMF5 TCR. Explicitly, DMF5 TCR mutations that enhance binding to the MART-1 peptide (αD26Y and βL98W) will be offset, or counterbalanced, by the TCR mutations that weaken binding with the MHC (αY50A or αY50V). In summary, the proposed net effect of this strategy in the DMF5 TCR will result in TCRs with an enhanced affinity towards the MART-1 peptide, but weakened affinity towards other, off-target, peptides presented in the context of HLA-A2.

The following five DMF5 mutant TCRs were generated: αD26Y, βL98W, αD26Y/βL98W, αD26Y/αY50A/βL98W, and αD26Y/αY50V/βL98W. The 3D affinities of
each TCR with the MART-1 9mer and 10mer peptides in the context of HLA-A2 were measured via SPR (Table 3). In general, TCR affinity against pMHC has been measured to range within 1-300 μM [399]. T cells that recognize self-antigens generally harbor TCRs with affinities ranging within 10-100 μM, while T cells that recognize foreign antigen harbor TCRs with affinities up to 1 μM [143, 400]. While implementing the structure-guided design strategy, it was of interest to generate TCRs that lie within the low micromolar range since this is similar to TCRs that recognize foreign peptides and result in full T cell activation. The 3D affinity of the WT DMF5 TCR measured against the MART-1 9mer/HLA-A2 complex and 10mer/HLA-A2 complex was 37 μM and 11 μM, respectively. These measurements fall within the range of TCRs that target self-antigens. The αD26Y TCR mutation enhanced the affinity of the TCR within this range against the MART-1 9mer and 10mer with the affinity measured to be 7 μM and 1.3 μM, respectively. The βL98W TCR mutation also enhanced the affinity compared to the WT TCR, with the affinity of the MART-1 9mer and 10mer measured to be 12 μM and 5.3 μM, respectively. When combined, the αD26Y/βL98W double mutant TCR further enhanced the affinity compared to the WT TCR, with MART-1 9mer and 10mer affinity measurements being 1.8 μM and 0.043 μM, respectively. This is notable because it is very rare to find natural TCRs with affinities in the nanomolar range, as the upper limit or threshold for optimal T cell function has been observed to fall within 1-5 μM [142, 351]. The αD26Y/αY50A/βL98W TCR reduced affinity compared to the WT TCR, with MART-1 9mer and 10mer affinities measured to be 228 μM and 36.4 μM, respectively.
Table 3. WT and Mutant DMF5 TCR-pMHC 3D Binding Affinities Measured via SPR. The average $K_D$ values of three independent experiments ± standard error of the mean are shown. 3D TCR affinity measurements were performed in the Baker lab.

<table>
<thead>
<tr>
<th>DMF5 TCR</th>
<th>MART-1 9mer (AAGIGILTV) $K_D$ (μM)</th>
<th>MART-1 10mer (ELAGIGILTV) $K_D$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>37 ± 3</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>$\alpha$D26Y</td>
<td>7 ± 2</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>$\beta$L98W</td>
<td>12 ± 1</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td>$\alpha$D26Y/$\beta$L98W</td>
<td>1.8 ± 0.3</td>
<td>0.043 ± 7</td>
</tr>
<tr>
<td>$\alpha$D26Y/$\alpha$Y50A/$\beta$L98W</td>
<td>228 ± 9</td>
<td>36.4 ± 7.1</td>
</tr>
<tr>
<td>$\alpha$D26Y/$\alpha$Y50V/$\beta$L98W</td>
<td>140 ± 7</td>
<td>16.1 ± 1.8</td>
</tr>
</tbody>
</table>

However, the affinity against the MART-1 10mer still falls within range of normal TCRs that recognize self-antigen, similar to the affinity of the WT TCR measured with the MART-1 9mer. Lastly, the $\alpha$D26Y/$\alpha$Y50V/$\beta$L98W TCR also had reduced affinity compared to the WT TCR, with MART-1 9mer and 10mer affinities measured to be 140 μM and 16.1 μM, respectively. Like the $\alpha$D26Y/$\alpha$Y50A/$\beta$L98W TCR, the affinity of the $\alpha$D26Y/$\alpha$Y50V/$\beta$L98W TCR with the MART-1 10mer falls within the upper range of normal TCRs that recognize self-antigen. These results implicate that as predicted, the DMF5 TCR mutations proposed to enhance binding to the MART-1 peptide, $\alpha$D26Y and $\beta$L98W, enhanced TCR 3D affinity, and the combination of these two mutations, $\alpha$D26Y/$\beta$L98W, drastically enhanced TCR 3D affinity compared to the WT DMF5 TCR. Furthermore, combining the $\alpha$D26Y/$\beta$L98W TCR mutations with a mutation that weakens TCR binding with the MHC, $\alpha$D26Y/$\alpha$Y50A/$\beta$L98W and $\alpha$D26Y/$\alpha$Y50V/$\beta$L98W, reduced TCR 3D affinity lower than the WT DMF5 TCR. Lastly, these 3D TCR affinity
measurements exemplify the effect of optimal peptide N-terminal anchor residues on TCR affinity. These 3D affinity measurements will be important in later sections in terms of their correlation to T cell function and cross-reactivity.

**WT vs. Mutant DMF5 TCR Recognition of Individual Alterations in the MART-1 Peptide**

We first wanted to determine how the structure-guided mutant DMF5 TCRs affected recognition of the MART-1 9mer peptide when single structural alterations were introduced, compared to the WT DMF5 TCR. The alanine scan assay is well established as a method to determine residues in a peptide that are important for TCR recognition [393, 401, 402]. We generated a panel of alanine substituted peptides based on the sequence of the MART-1 9mer peptide (AAGIGILTV). If an alanine was already present at a residue, an isoleucine was substituted instead. Herein, we could determine how mutations in the DMF5 TCR altered recognition of alanine substituted MART-1 peptides.

We transduced Jurkat E6.1 cells to express the WT or each mutant DMF5 TCR. Jurkat E6.1 cells were used here to eliminate the donor variability generally associated with polyclonal PBL-derived T cells. Transduction efficiencies four days after transduction are shown in Figure 14. The data indicates that initial Jurkat E6.1 populations were transduced between 13% and 18% (based on our marker of transduction, CD34). Jurkat E6.1 cells express minimal levels of endogenous CD34, as shown at 5.06%. TCR transduced Jurkat E6.1 cells were subsequently enriched for high and uniform CD34 expression using anti-CD34 immunomagnetic beads. CD34 surface expression after CD34 enrichment is shown in Figure 15. The data indicate that after
CD34 enrichment, Jurkat E6.1 populations exhibited between 86% and 97% CD34 surface expression. We did not stain for TCR surface expression via TCR alpha or TCR beta chain mAbs because this quantification would include expression of mispaired TCRs. Furthermore, we did not stain for TCR surface expression via tetramers or dextramers because it is possible that the introduced TCR mutations could affect this quantification. However, previous studies indicated that CD34 surface expression correlated with TCR surface expression in a linear fashion [94]. Figure 15 depicts an example of the uniform CD34⁺ cell populations that were obtained and used for functional assays after transduction and CD34 enrichment. In this dissertation, this method of CD34 enrichment, followed by confirmation of uniform and high CD34 expressing populations, was completed each time a transduction generated newly TCR transduced cells (in both Jurkat E6.1 cells and T cells). These CD34 purified TCR transduced Jurkat E6.1 cells were used as effector cells and cocultured with T2 cells loaded with either the WT MART-1 9mer peptide or an alanine substituted peptide. The amount of IL-2 produced against each alanine substituted peptide was normalized to the amount of IL-2 produced against the MART-1 9mer peptide for each DMF5 TCR, respectively. This was done in order to elucidate the residues important for recognition relative to recognition of the MART-1 9mer peptide, and to easily observe alterations in the patterns of recognition between each of the DMF5 TCRs (Figure 16).
Figure 14. CD34 Surface Expression on Jurkat E6.1 Cells Pre-CD34 Enrichment. Four days after retroviral transduction, untransduced and TCR transduced Jurkat E6.1 cells were labeled with an anti-CD34 mAb to measure the transduction efficiency of each population.
Figure 15. CD34 Surface Expression on Jurkat E6.1 Cells Post-CD34 Enrichment. Six days after retroviral transduction, and two days after CD34 immunomagnetic bead enrichment, TCR transduced Jurkat E6.1 cells were labeled with an anti-CD34 mAb to measure the CD34 enrichment efficiency of each population. As a result, the data indicate high and uniform CD34 expression among the different populations.
Figure 16. Impact of DMF5 TCR Mutations on Recognition of Alanine Substituted MART-1 9mer Peptides. Jurkat E6.1 cells expressing either the WT TCR or each mutant DMF5 TCR were stimulated with T2 cells loaded with the WT MART-1 9mer peptide (AAGIGILTV) or alanine substituted peptides for 18 hours. IL-2 release was measured by ELISA in triplicate wells. Fold change in reactivity was normalized within each TCR to reactivity against the WT MART-1 9mer peptide, respectively. Average MART-1 9mer reactivity was 913 pg/mL. Data are the average of three independent experiments and error bars represent the standard error of the mean from three independent experiments.

We first observed the pattern of recognition elicited by the WT DMF5 TCR before determining how the structure-guided DMF5 TCR mutants affected recognition of alanine substituted MART-1 9mer peptides. Alanine substitutions at positions 3 through 6 and position 8 of the MART-1 peptide eliminated reactivity in Jurkat E6.1 cells expressing the WT DMF5 TCR, indicating these residues are critical for WT DMF5 recognition of the MART-1 9mer peptide. Additionally, substitutions at positions 2 and 7 reduced reactivity to 14% and 26% of reactivity compared to the MART-1 9mer peptide,
respectively, but did not completely eliminate reactivity, indicating these two peptide residues are important but not critical for recognition of the MART-1 9mer peptide. In summary, positions 3 through 6, and 8 of the MART-1 9mer peptide are critical for recognition by Jurkat E6.1 cells expressing the WT DMF5 TCR.

After determining how Jurkat E6.1 cells expressing the WT DMF5 TCR recognized alanine substituted MART-1 9mer peptides, we determined how mutations in the DMF5 TCR altered this pattern of recognition. The pattern of recognition observed with the WT DMF5 TCR did not hold true in Jurkat E6.1 cells expressing the αD26Y TCR. In cells expressing the αD26Y TCR, substitutions at positions 4 and 6 reduced reactivity to that of about 13% and 8% of reactivity against the MART-1 9mer peptide, respectively. This pattern is different compared to the WT TCR, as now substitutions at positions 2, 3, 5, 7, and 8 are tolerated. Despite this tyrosine mutation at position 26 of the TCR alpha chain targeting the N-terminal region of the MART-1 peptide, the αD26Y TCR allowed for enhanced reactivity relative to the MART-1 9mer peptide at positions 7 and 8. These results indicated the tyrosine TCR mutation enhances binding with the N-terminal region of the peptide, or the pMHC complex, substantially enough, to where the TCR is tolerant of alterations at the C-terminal end of the MART-1 9mer peptide. Overall, the αD26Y TCR modification designed to enhance binding to the N-terminal region of the MART-1 peptide also impacted recognition of MART-1 9mer peptides containing alanine substitutions at the C-terminal end.

In the next DMF5 TCR mutant, Jurkat E6.1 cells expressing the βL98W TCR displayed a pattern fairly similar to that of the WT TCR. One exception is the βL98W TCR mutation now allows for recognition of alanine substitution at position 7, equal to
that of the MART-1 9mer peptide. This indicated the tryptophan mutation at position 98 of the DMF5 TCR beta chain is not specific for the leucine at position 7 of the MART-1 9mer peptide, as it tolerated an alanine substitution. Furthermore, the βL98W TCR was more tolerant of alanine substitution at position 8 of the MART-1 9mer peptide compared to the WT TCR. This indicated that the tryptophan TCR mutation could be enhancing binding with the peptide beyond position 7. In summary, the βL98W TCR is not as tolerant with alanine substitutions as the αD26Y TCR, but also is possibly not as specific for position 7 in the MART-1 peptide as initially proposed.

After determining how the single positive DMF5 TCR mutants altered the recognition pattern of alanine substituted MART-1 9mer peptides, we determined how the combination of these DMF5 TCR mutations altered peptide recognition. The combination of the αD26Y and βL98W TCR mutations in the αD26Y/βL98W TCR allowed for the most promiscuous pattern of recognition of alanine substituted MART-1 9mer peptides compared to all of the TCRs. The observed pattern resembled a pattern of recognition most similar to the αD26Y TCR. Only alanine substitution at positions 4 and 6 resulted in reduced reactivity relative to reactivity against the MART-1 9mer peptide. This suggested the combination of the αD26Y and βL98W TCR mutations enhanced binding to the pMHC, as the TCR now allows for recognition of an alanine at position 4 in the MART-1 9mer. With the αD26Y/βL98W TCR, this recognition of alanine substitution at position 4 was 57% of the MART-1 9mer peptide, compared to about 13% and 0% by the αD26Y and βL98W TCRs, respectively, indicating more than an additive effect of the two TCR mutations (57% > 13% + 0%). Overall, these results suggested the enhanced affinity and structural changes of the αD26Y/βL98W TCR
mutations allow it to be more permissive in recognition of alanine substituted MART-1 9mer peptides compared to the αD26Y, βL98W, and WT DMF5 TCRs.

Our results thus far indicated that single or double positive TCR mutations in the DMF5 TCR altered the recognition pattern of alanine substituted MART-1 9mer peptides compared to the WT DMF5 TCR. We next determined how these patterns were affected by the inclusion of a negative mutation, one that eliminated binding with three residues in the MHC, to the αD26Y/βL98W TCR. When a TCR mutation that eliminated binding to three residues in the MHC was added to the two positive TCR mutations, Jurkat E6.1 cells expressing the αD26Y/αY50A/βL98W TCR exhibited a pattern similar to the WT DMF5 TCR. One minor difference is a slight enhancement in relative recognition when position 7 is alanine substituted, 52% compared to 26% respectively, relative to reactivity against the MART-1 9mer peptide. This was most likely due to the presence of the βL98W TCR mutation which was designed to enhance binding with the leucine at position 7. Additionally, alanine substitution at position 1 was very well tolerated, which could be attributed to the strength of the αD26Y and βL98W TCR mutations despite presence of the αY50A TCR mutation. In summary, these results suggested the low 3D affinity (228 μM against the MART-1 9mer) and structural changes of the αD26Y/αY50A/βL98W TCR mutations do not significantly affect the pattern of recognition of alanine substituted MART-1 9mer peptides compared to the WT TCR.

Lastly, we observed how recognition patterns were affected by the inclusion of a different negative mutation, one that weakened binding with three residues in the MHC (but did not eliminate binding like αY50A), to the αD26Y/βL98W TCR. Jurkat E6.1 cells expressing the αD26Y/αY50V/βL98W TCR exhibited a pattern of recognition very
similar to the βL98W TCR. This indicated that the addition of α50V and even more so, α50A, to the αD26Y/βL98W TCR, counterbalanced the permissive reactivity. Specifically, recognition of alanine substituted position 7 was comparable to reactivity against the MART-1 9mer peptide, as was the case with the βL98W TCR. Albeit, reduced relative reactivity was observed at alanine substituted position 7 with the αD26Y/αY50A/βL98W TCR, indicating the αY50A mutation better offset enhanced promiscuity at this residue than the αY50V mutation. However, aside from position 7, the patterns of recognition observed with the αD26Y/αY50A/βL98W TCR and the αD26Y/αY50V/βL98W TCR closely resemble the pattern observed with the WT TCR, indicating the addition of the mutations that weaken binding with the MHC, can counterbalance the promiscuity observed with the αD26Y and αD26Y/βL98W TCRs. Overall these results suggested that each MART-1 targeting TCR mutation did not increase MART-1 specificity, but combination with a TCR mutation that weakens binding with the MHC can enhance target specificity.

**Impact of Mutant DMF5 TCRs on On-target Tumor Killing**

The intention for designing these TCRs was to study TCR/pMHC interactions and furthermore, eventually to use them therapeutically to treat melanoma. Therefore, before moving forward with functional T cell assays, we wanted to confirm T cells expressing the modified DMF5 TCRs could elicit killing of HLA-A2+ melanoma cells. A summary of the DMF5 TCRs and their measured affinities with the MART-1 9mer peptide/HLA-A2 is shown in Table 4. If affinity correlates to targeted killing, based on the measured 3D affinities, we would expect that cells expressing the αD26Y/βL98W TCR would elicit the highest level of targeted tumor killing, while cells expressing the
αD26Y/αY50A/βL98W TCR would elicit the lowest level of targeted tumor killing. An LDH cytotoxicity assay was performed using human T cells transduced to express either the WT DMF5 TCR or each mutant DMF5 TCR and an HLA-A2+/MART-1+ melanoma tumor, MEL 624-28, and an HLA-A2+/MART-1+ melanoma tumor, MEL 624 (Figure 17). The percentage of TCR transduced T cells used in these experiments ranged between 84% and 89% among the different DMF5 TCR expressing T cell populations. Among the TCR transduced T cell populations, the proportion of CD4+ T cells ranged between 27% and 35% and the proportion of CD8+ T cells ranged between 65% and 73%. These minor differences in the percentages of TCR transduced T cells and CD4+ to CD8+ T cell ratios had no correlation with differences observed in targeted cytotoxicity. All killing by the WT and mutant DMF5 TCR transduced T cells was HLA-A2 restricted, as there was no killing observed with HLA-A2- MEL 624-28 (Figure 17). T cells expressing each of the mutated TCRs exhibited equal or better killing of MEL 624 than T cells expressing the WT TCR. Specifically, T cells expressing the WT DMF5 TCR exhibited 37% cytotoxicity of MEL 624 tumor cells. T cells expressing the αD26Y TCR or the αD26Y/βL98W TCR exhibited statistically significantly more MEL 624 killing (65% and 61%, respectively) compared to T cells expressing the WT TCR. Lastly, T cells expressing the βL98W TCR, αD26Y/αY50A/βL98W TCR, or αD26Y/αY50V/βL98W TCR, exhibited MEL 624 tumor killing at similar or increased levels (48%, 39%, and 52%, respectively) compared to T cells expressing the WT TCR.
### Table 4. Summary of Structure-Guided DMF5 TCR Mutations

<table>
<thead>
<tr>
<th>DMF5 TCR</th>
<th>TCR Mutation Target</th>
<th>CDR Region of TCR Modifications</th>
<th>$K_D$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>αD26Y/βL98W</td>
<td>N and C terminal end of MART-1</td>
<td>CDR1, CDR3</td>
<td>1.8 μM</td>
</tr>
<tr>
<td>αD26Y</td>
<td>N terminal end of MART-1</td>
<td>CDR1</td>
<td>7 μM</td>
</tr>
<tr>
<td>βL98W</td>
<td>C terminal end of MART-1</td>
<td>CDR3</td>
<td>12 μM</td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td>37 μM</td>
</tr>
<tr>
<td>αD26Y/αY50V/βL98W</td>
<td>N and C terminal end of MART-1, and MHC</td>
<td>CDR1, CDR2, CDR3</td>
<td>140 μM</td>
</tr>
<tr>
<td>αD26Y/αY50A/βL98W</td>
<td>N and C terminal end of MART-1, and MHC</td>
<td>CDR1, CDR2, CDR3</td>
<td>228 μM</td>
</tr>
</tbody>
</table>

The $K_D$ values indicated were measured in the MART-1 nonameric peptide. DMF5 TCR mutations in green indicate positive, peptide targeting mutations. DMF5 TCR mutations in red indicate negative, MHC weakening mutations. 3D TCR affinity measurements were completed in the Baker lab.
Figure 17. Impact of DMF5 TCR Mutations on Melanoma Killing. Human untransduced or TCR transduced T cells (with either the WT TCR or a mutant DMF5 TCR) were cocultured with tumor cells for 18 hours in a 1:1 effector:target ratio. MEL 624-28 tumor cells were used as an HLA-A2/MART-1+ melanoma control. MEL 624 tumor cells were used as an HLA-A2+/MART-1+ melanoma line. Data represent the mean of two independent experiments and error bars represent the standard error of the mean from two independent experiments. One representative donor is shown. ***P < 0.001; **P < 0.01; *P < 0.05 when compared to the percent MEL 624 killing by the WT TCR by two-way ANOVA using Sidak’s multiple comparisons test.

Based on 3D affinity, it was unexpected that T cells expressing the αD26Y/αY50A/βL98W or αD26Y/αY50V/βL98W TCR exhibited equal or better MEL 624 killing compared to T cells expressing the WT TCR. However, it has been demonstrated that the targeted release of lytic granules only requires engagement of three TCR/pMHC interactions [152, 403]. This suggests TCR affinity could have less of an influence on lytic activity in T cells. This could further be supported by the observation
that despite the higher TCR affinity (roughly 3-fold higher) of the βL98W TCR, it did not elicit significantly more MEL 624 killing than the WT TCR. Overall, these results demonstrated T cells expressing each of the modified DMF5 TCRs could elicit HLA-A2 restricted melanoma killing and 3D TCR affinity does not directly correlate with target cell lysis.

**Impact of Mutant DMF5 TCRs on Cross-Reactivity**

We next sought to determine how the structure-guided DMF5 TCR mutations altered cross-reactivity in T cells, beyond alanine substituted peptides, compared to the WT DMF5 TCR. We utilized a panel of previously described peptides that were selected because they are homologous with the MART-1 9mer peptide [327, 328]. These peptides and their sequence homology with the MART-1 9mer are shown in Table 5. Recurring residues include glycine, alanine, valine, and leucine, as they are featured extensively in the MART-1 9mer epitope. Many of the selected peptides conform to the central [G, A, V]xGx motif at positions 3 through 6 of the MART-1 9mer epitope. The panel is comprised of both self- and non-self-peptides that are naturally occurring and physiologically relevant. Previous studies demonstrated MART-1 reactive T cells could lyse target cells loaded with some, but not all, of the peptides in this selected panel [327]. Therefore, we decided to perform preliminary screenings for cross-reactivity to determine which MART-1 homologs elicited a significant response from T cells expressing the WT TCR or each of the modified DMF5 TCRs (Figure 18). Specifically, TCR transduced T cells expressing either the WT TCR or each mutant DMF5 TCR were cocultured with T2 cells loaded with the MART-1 9mer peptide or each MART-1 homolog.
Table 5. MART-1 Homolog Peptide Panel. Homology with the MART-1 9mer is indicated in black [327]. ● indicates self-peptide. The following abbreviations are used throughout the text: Human receptor expression enhancing protein 5 (HREEP 5) and G-protein coupled receptor 3 (GPCR 3).
Figure 18. Example of Initial Screen for Cross-Reactivity. An example of initial cross-reactivity screening shown with TCR transduced T cells expressing the αD26Y DMF5 TCR. For screening, human T cells expressing either WT or mutant DMF5 TCR stimulated with T2 cells loaded with the HCV NS3 peptide (KLVALGINAV), MART-1 9mer peptide (AAGIGILTV), or MART-1 homologs. IFN-γ release was measured by ELISA in triplicate wells. Antigen reactivity is defined as a T cell culture that secretes twice background, or twice the amount of IFN-γ production as with unpulsed T2 cells, and over 200 pg/mL. One representative experiment from one representative donor is shown.

The amount of IFN-γ produced against each peptide loaded target was measured. The representative example shown in Figure 18 depicts antigen reactivity by T cells expressing the high affinity αD26Y DMF5 TCR. Here, antigen reactivity is defined as a T cell culture that secretes twice background, or twice the amount of IFN-γ production as with unpulsed T2 cells, and over 200 pg/mL. It is evident that αD26Y TCR expressing T
cells recognized target cells loaded with multiple MART-1 homologs. Specifically, in this
donor and experiment, αD26Y TCR expressing T cells recognized target cells loaded
with HSV-1 glycoprotein III, human CD9, M. tuberculosis protein I, ADP-ribose
diphosphatase, and GPCR 3. Furthermore, there are many MART-1 homolog loaded
targets that were not recognized. This is concurrent with previous findings in that
despite sequence homology with the MART-1 9mer peptide, not all MART-1 homolog
loaded target cells elicited a response from MART-1 reactive T cells [327]. This
experiment was repeated six times with T cells expressing the WT TCR or each of the
mutant DMF5 TCRs (two independent experiments in three different healthy donors).
For all future functional experiments, we decided to concentrate on the MART-1
homologs that elicited a T cell response from any of the DMF5 TCRs in any donor or
experiment. Specifically, a T cell response was defined as any MART-1 homolog that
elicited IFN-γ production greater than with T2 cells alone (Table 6). It was interesting to
observe that our results obtained with the alanine scan assay did not completely
coincide with the MART-1 homologs that were recognized. For example, results from
the alanine scan assay (Figure 16) indicated the importance of positions 3 through 6
and 8 in the MART-1 9mer peptide for recognition by WT TCR expressing cells, but
many MART-1 homologs with mutations at one or more of these peptide residues were
recognized (all of the MART-1 homologs in Table 6). Conversely, MART-1 homologs
that maintained sequence homology at one or more of these peptide residues were not
recognized. For example, the S. cerevisiae protein I peptide (in Table 5) differs from the
MART-1 9mer at four residues, but the altered residues are still very hydrophobic, and
sequence homology remains at the central peptide positions, 4 through 6.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>● MART-1 9-mer</td>
<td>AAGIGILTV</td>
</tr>
<tr>
<td>● MART-1 10-mer</td>
<td>ELAGIGILTV</td>
</tr>
<tr>
<td>● Human CD9</td>
<td>AVGIGIAVV</td>
</tr>
<tr>
<td>● Human elongation factor 1α</td>
<td>IGGIGTVPV</td>
</tr>
<tr>
<td>● G-protein coupled receptor 3</td>
<td>ALGLGLLPV</td>
</tr>
<tr>
<td>● Human receptor expression enhancing protein 5</td>
<td>LGVIGLVAL</td>
</tr>
<tr>
<td>M. tuberculosis protein II</td>
<td>IAGPGTITL</td>
</tr>
<tr>
<td>M. tuberculosis protein I</td>
<td>LGGLGLFFA</td>
</tr>
<tr>
<td>β-endoxylanase</td>
<td>GAGIGVLTA</td>
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<td>ADP-ribose diphosphatase</td>
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<tr>
<td>HSV-2 glycoprotein F</td>
<td>GAGIGVAVL</td>
</tr>
<tr>
<td>HSV-1 glycoprotein III</td>
<td>IAGIGILAI</td>
</tr>
</tbody>
</table>

Table 6. MART-1 Homolog Peptide Panel Used In Functional Assays. Homology with the MART-1 9mer is indicated in black [327]. ● indicates self-peptide.
However, T cells expressing the WT TCR or modified DMF5 TCRs did not recognize T2 cells loaded with this peptide in any experiment. It would be plausible to hypothesize that cross-reactive epitopes have a better N-terminal anchor since alanine, the N-terminal anchor in the MART-1 9mer peptide, is generally considered a weak anchor residue. It has been demonstrated that peptides conform differently in the MHC depending upon their anchor residues [404, 405]. However this was not the case with the S. cerevisiae protein I peptide, as it has a leucine N-terminal anchor and was not recognized. Furthermore, cross-reactivity was observed against MART-1 homologs with an N-terminal alanine anchor residue (M. tuberculosis protein II, β-endoxylanase, ImrA, HSV-2 glycoprotein F, HSV-1 glycoprotein III) and conversely, no cross-reactivity was observed against some MART-1 homologs with N-terminal leucine anchors (E. coli peptide, P. putida, Measles Virus, S. gordonii). Overall, altered-peptide ligands that have sequence homology with the MART-1 9mer peptide could be recognized despite mutations at residues previously implicated as imperative for MART-1 9mer peptide recognition in the alanine scan. These results indicated that the alanine scan assay can be a suitable method for making a general prediction about the importance of an individual residue in the MART-1 9mer peptide for recognition. However, it does not take into account the effects or contributions of alterations in the peptide with different amino acids (other than alanine) and/or the combination of multiple alterations in the peptide. Overall, cross-reactivity against a given peptide is probably more dependent upon the net effect or, the overall structure and antigenic surface of the peptide in the HLA-A2 complex, versus individual mutations at a given epitope.
We next determined how human T cells expressing each mutant DMF5 TCR altered MART-1 specificity and recognition of the MART-1 homologs compared to T cells expressing the WT DMF5 TCR in terms of polyfunctional T cell responses. WT TCR and mutant DMF5 TCR transduced T cells were stimulated with T2 cells loaded with the MART-1 peptide or the MART-1 homologs (Table 6). The typical functional readout in the field for T cell function is IFN-γ production. However, it is known that T cells are capable of more complex functional phenotypes and have been shown to be polyfunctional [95, 406-411]. Furthermore, analysis of intracellular IFN-γ production from PBL-derived T cells from stage IV melanoma patients who received therapeutic vaccination, revealed no correlation to survival [412]. Additionally, multiple T cell subtypes have been described as important in tumor and disease clearance [413-416]. Therefore, since polyfunctionality could be important for on-target and off-target recognition, we examined surface expression of CD107A as a surrogate marker for lysis [417, 418], and intracellular expression of IFN-γ, TNF-α, IL-17A, IL-2, IL-4, and IL-22 following antigen stimulation on a per cell basis. This cytokine panel includes Th1, Th2, and Th17 cytokines.

We first focused on antigen recognition in CD4+ T cells because true cross-reactivity, as a result of changes in the TCR/pMHC interface, are highlighted in the CD4+ T cells due to the absence of assistance in signaling and stabilization from the CD8 co-receptor [92]. Furthermore, the DMF5 TCR mutations designed using the structure-guided approach did not take into account CD8. The gating strategies used for all experiments are shown in Figures 4-6.
We first observed antigen specificity and cross-reactivity of CD4⁺ T cells expressing the WT DMF5 TCR. Here, the percentage of antigen reactive T cells expressed any one or more functional marker (CD107A or cytokines). The percentages of antigen reactive T cells were determined after respective background subtraction, as described in Chapter Two. The average of six experiments found 19% of CD4⁺ T cells expressing the WT DMF5 TCR were reactive against MART-1 9mer loaded targets (Figure 19). The MART-1 10mer has a higher binding affinity for HLA-A2 [392], however, compared to the MART-1 9mer, the percentages of reactive T cells were similar (22% vs. 19%). This indicated that MART-1 peptide binding stability did not significantly affect the percentage of antigen reactive CD4⁺ T cells. Recognition of MART-1 homologs by CD4⁺ T cells expressing the WT TCR was low, between 0.15% and 1.8%, indicating CD4⁺ WT DMF5 TCR expressing T cells are barely reactive against the MART-1 homologs. In conclusion, CD4⁺ T cells expressing the WT DMF5 TCR are MART-1 reactive, but appear to be minimally cross-reactive.

We extended our analysis to the structure-guided mutant DMF5 TCRs after determining the percentages of MART-1 and MART-1 homolog reactive WT DMF5 TCR expressing CD4⁺ T cells. Since the αD26Y TCR mutation enhanced binding with the MART-1 peptide, we would expect an increase in the percentages of MART-1 reactive T cells. Although not statistically significant, as predicted, CD4⁺ T cells expressing the αD26Y TCR displayed an increase in the percentage of MART-1 9mer reactive T cells (27%) compared to WT TCR expressing T cells (19%). This result indicated the αD26Y TCR mutation enhanced binding with MART-1.
Figure 19. Impact of DMF5 TCR Mutations in TCR Transduced CD4+ T Cells against MART-1 and MART-1 Homologs. Human TCR transduced T cells were stimulated with peptide loaded T2 cells for 5 hours and then stained for cell surface CD3, CD4, CD8, CD34, CD107A, and intracellular IFN-γ, TNF-α, IL-17A, IL-2, IL-4, and IL-22. Subsequent percent antigen reactive T cells were calculated by subtracting the percentage of non-reactive TCR transduced CD4+ T cells from 100. As described in Chapter Two, antigen reactivity was determined after background subtraction (specifically, subtraction of background immunofluorescence with HCV loaded T2 cells). Non-reactive TCR transduced CD4+ T cells did not express CD107A or cytokines. The reactivity of CD3+CD34+CD4+ TCR transduced T cells is shown. Error bars represent the standard error of the mean from three donors, two independent repeats (6 experiments). Self-peptides are indicated by a black triangle. **** P <0.0001; ***P < 0.001; **P < 0.01; *P < 0.05 when the percentage of antigen reactive T cells against each peptide with a given TCR was compared to the percentage of antigen reactive T cells to the respective peptide with the WT TCR by two-way ANOVA using Sidak’s multiple comparisons tests.

However, compared to T cells expressing the WT TCR, T cells expressing the αD26Y TCR demonstrated an increase in the percentages of MART-1 homolog reactive T cells (0.15% - 1.8% vs. 2.4% - 26%), reaching statistical significance with HSV-1 glycoprotein III and M. tuberculosis protein I (0.7% and 0.9% vs. 29% and 22%). Overall, the tyrosine
TCR mutation at position 26 in the TCR alpha chain did not increase MART-1 specificity.

We next observed how the introduction of a different MART-1 peptide targeting TCR mutation, βL98W, affected MART-1 and MART-1 homolog recognition in CD4+ T cells. Although not significant, as predicted, CD4+ T cells expressing the βL98W TCR displayed an increase in the percentage of MART-1 9mer reactive T cells (23%) compared to WT TCR expressing T cells (19%). Furthermore, recognition of MART-1 homologs by T cells expressing the βL98W TCR was low, between 0.4% and 5.9%. This range was slightly greater than the percentage of MART-1 homolog reactive T cells expressing the WT TCR, but no statistically significant differences were observed. This indicated the tryptophan TCR mutation at position 98 of the DMF5 TCR beta chain did not significantly affect recognition of MART-1 or MART-1 homologs compared to the WT TCR. We next observed the effect of the combination of the αD26Y TCR and βL98W TCR mutations on antigen specificity and cross-reactivity. T cells expressing the αD26Y/βL98W TCR demonstrated a reduction in the percentage of MART-1 9mer reactive T cells compared to the WT TCR (11.6% vs. 19%). Since the 3D affinity of this TCR was measured to be 1.8 μM against the MART-1 9mer, this result was not surprising, as it has been demonstrated when a TCR’s affinity approaches 1 μM, a decline in TCR signaling and in T cell function is observed [142, 145, 419]. Compared to T cells expressing the WT TCR, an increase in the percentages of MART-1 homolog reactive T cells was observed with the αD26Y/βL98W TCR, reaching statistical significance with HSV-1 glycoprotein III and M. tuberculosis protein I loaded targets (0.7% and 0.9% vs. 10% and 10%). Overall, these results suggested the combination of
the tyrosine TCR mutation at position 26 in DMF5 TCR alpha chain and the tryptophan TCR mutation at position 98 in the TCR beta chain did not increase MART-1 specificity and attenuated MART-1 specific T cell reactivity.

We next evaluated the effect of the introduction of a MHC weakening TCR mutation on antigen specificity and cross-reactivity. There was a statistically significant reduction in the percentage of MART-1 9mer reactive T cells expressing the αD26Y/αY50A/βL98W TCR compared to the WT TCR, 8% vs. 19%, but this was not surprising based on its reduced 3D TCR affinity. The percentages of MART-1 homolog reactive T cells was comparable between T cells expressing the αD26Y/αY50A/βL98W TCR and the WT TCR (0.6% - 2.1% vs. 0.15% - 1.8%). This indicated the addition of the αY50A TCR mutation to the αD26Y/βL98W TCR restored the percentage of MART-1 homolog reactive T cells to WT TCR levels. Compared to the introduction of the αY50A TCR mutation, the introduction of the αY50V TCR mutation had less of an effect on MART-1 and MART-1 homolog recognition. Specifically, T cells expressing the αD26Y/αY50V/βL98W TCR exhibited a similar percentage of MART-1 9mer reactive T cells compared to T cells expressing the WT TCR (21% vs. 19%). Furthermore, aside from minor increases, (0.46% - 4.8% vs. 0.15% - 1.8%), the percentages of MART-1 homolog reactive CD4+ T cells was comparable between T cells expressing the αD26Y/αY50V/βL98W TCR and the WT TCR. These results were unexpected considering the measured 3D TCR affinity of the αD26Y/αY50V/βL98W TCR was lower compared to the WT TCR. In summary, compared to the WT TCR, the percentages of cross-reactive CD4+ T cells was enhanced with the αD26Y TCR and the αD26Y/βL98W TCR, and the percentages of MART-1 9mer reactive CD4+ T cells was reduced with the
αD26Y/βL98W TCR and the αD26Y/αY50A/βL98W TCR. Overall, none of the DMF5 TCR mutations enhanced MART-1 specificity in TCR transduced CD4+ T cells.

TCR affinity has been correlated to antigen specificity and cross-reactivity and has been implicated to be of importance in T cell function. Thus, it was important to determine how the MART-1 and MART-1 homolog recognition by the modified DMF5 TCRs correlated to their 3D affinity. It is generally well appreciated that higher affinity TCRs are more cross-reactive [179, 420]. Based solely on this concept, we would have expected to observe an increase in the percentages of MART-1, and possibly MART-1 homolog, reactive CD4+ T cells in T cells transduced to express the TCRs whose 3D affinity was higher than the WT TCR (αD26Y TCR, βL98W TCR, and αD26Y/βL98W TCR). There was no significant increase (but trending towards) in the percentages of MART-1 reactive CD4+ T cells expressing the αD26Y TCR or βL98W TCR, compared to T cells expressing the WT TCR. However, T cells expressing the αD26Y TCR or αD26Y/βL98W TCR did exhibit a significant increase in the percentage of antigen reactive CD4+ T cells against two MART-1 homologs (HSV-1 glycoprotein III and M. tuberculosis protein I), coinciding with their increased 3D TCR affinity. There were no significant differences observed with T cells expressing the βL98W TCR compared to the WT TCR despite the 3-fold 3D TCR affinity enhancement of the βL98W TCR. However, minor increases were observed. Based solely on 3D affinity, we would have expected a decrease in the percentage of MART-1 and MART-1 homolog reactive CD4+ T cells in T cells transduced to express TCRs whose 3D affinity was lower than the WT TCR (αD26Y/αY50A/βL98W and αD26Y/αY50V/βL98W). A significant decrease in the percentage of MART-1 reactive CD4+ T cells was observed in T cells expressing the
aD26Y/αY50A/βL98W TCR compared to the WT TCR, coinciding with the decrease in 3D TCR affinity. However, the percentage of MART-1 and MART-1 homolog reactive CD4+ T cells in T cells expressing the aD26Y/αY50V/βL98W TCR was similar to that of T cells expressing the WT TCR, aside from slight, non-significant increases against a few MART-1 homologs (ADP-ribose diphosphatase, HREEP 5, human elongation factor 1α, and HSV-2 glycoprotein F). In summary, these results suggested the aD26Y/αY50V/βL98W TCR’s reduced 3D affinity does not directly correlate to the observed T cell specificity. However, 3D affinity generally correlated to the percentages of TCR transduced MART-1 reactive and cross-reactive CD4+ T cells among the WT TCR and modified DMF5 TCRs.

The analysis of the WT DMF5 TCR and mutant DMF5 TCR transduced CD4+ T cells allowed us to examine how the structure of the TCR/pMHC influenced T cell function and cross-reactivity. However, it has been implicated that the CD8 co-receptor can affect antigen specificity and cross-reactivity through stabilization of the TCR/pMHC and through T cell signaling [398, 421, 422]. Therefore, we next determined how recognition of MART-1 and the MART-1 homologs was altered in WT TCR and mutant DMF5 TCR transduced CD8+ T cells. We first observed antigen specificity and cross-reactivity of CD8+ T cells expressing the WT DMF5 TCR. The average of six experiments found 44% of CD8+ T cells expressing the WT DMF5 TCR were reactive against MART-1 9mer loaded targets (Figure 20). Contrary to CD4+ T cells, CD8+ T cells expressing the WT TCR recognized nine MART-1 homolog loaded targets, ranging from 1.4% to 18% of WT DMF5 TCR transduced T cells. Some of these recognized MART-1 homologs were self-peptides (HREEP 5, human elongation factor 1α, and
human CD9). In summary WT DMF5 TCR expressing CD8+ T cells were more reactive against MART-1 and MART-1 homolog loaded targets than the CD4+ T cells. These results implicated the role of CD8 in cross-reactivity.

After determining how CD8+ T cells expressing the WT DMF5 TCR recognized MART-1 and MART-1 homologs, we determined how mutations in the DMF5 TCR altered reactivity in TCR transduced CD8+ T cells. Since the αD26Y TCR and βL98W TCR mutations enhanced binding with the MART-1 peptide, we would expect an increase in the percentages of MART-1 reactive T cells. However, neither of the TCR transduced T cell populations expressing TCRs harboring mutations that enhanced binding to MART-1 (αD26Y TCR and βL98W TCR) demonstrated increased percentages of MART-1 9mer reactive T cells compared to T cells expressing the WT TCR (42% and 42% vs. 43%). These results indicate CD8 can minimize the targeted effects of these mutations. More specifically, the contribution of the CD8 co-receptor in T cell function appears to overshadow the minor impact of the positive TCR mutations. This observation indicated the structure-guided strategy can have different effects in CD4+ and CD8+ T cells. As seen in the TCR transduced CD4+ T cells, the percentage of MART-1 homolog reactive T cells was increased in T cells expressing the αD26Y TCR compared to the WT TCR, with statistical significance reached with HSV-1 glycoprotein III (44%), M. tuberculosis protein I (34%), and GPCR 3 (23%). With the second MART-1 targeting TCR mutation, βL98W, the pattern of antigen reactivity against the MART-1 homologs was similar between T cells expressing the βL98W TCR and the WT TCR.
Figure 20. Impact of DMF5 TCR Mutations in TCR Transduced CD8+ T Cells against MART-1 and MART-1 Homologs. Human TCR transduced T cells were stimulated with peptide loaded T2 cells for 5 hours and then stained for cell surface CD3, CD4, CD8, CD34, CD107A, and intracellular IFN-γ, TNF-α, IL-17A, IL-2, IL-4, and IL-22. Subsequent percent antigen reactive T cells were calculated by subtracting the percentage of non-reactive TCR transduced CD8+ T cells from 100. As described in Chapter Two, antigen reactivity was determined after background subtraction (specifically, subtraction of background immunofluorescence with HCV loaded T2 cells). Non-reactive TCR transduced CD8+ T cells did not express CD107A or cytokines. The reactivity of CD3+CD34+CD8+ TCR transduced T cells is shown. Error bars represent the standard error of the mean from three donors, two independent repeats (6 experiments). Self-peptides are indicated by a black triangle. **** P <0.0001; ***P < 0.001; **P < 0.01; *P < 0.05 when the percentage of antigen reactive T cells against each peptide with a given TCR was compared to the percentage of antigen reactive T cells to the respective peptide with the WT TCR by two-way ANOVA using Sidak’s multiple comparisons tests.

However, although not statistically significant, an increase in the percentage of human CD9 reactive T cells was observed in T cells expressing the βL98W TCR, compared to the WT TCR (9.4% vs. 3%). With the βL98W TCR mutation, we would expect enhanced reactivity against the four MART-1 homologs that contain a leucine at position 7 (GPCR 3, β-endoxylanase, ADP-ribose diphosphatase, and HSV-1 glycoprotein III). This was not observed, indicating the tryptophan is unable to differentiate a leucine from other
hydrophobic residues at position 7 of the 9mer peptides. We next observed the effect of the combination of the αD26Y TCR and βL98W TCR mutations on antigen specificity and cross-reactivity in TCR transduced CD8+ T cells. T cells expressing the αD26Y/βL98W TCR exhibited a significant decrease in MART-1 reactive T cells compared to the WT TCR (16% vs. 44%). This again, coincides with this TCR’s supraphysiological high affinity. Compared to T cells expressing the WT TCR, expression with the αD26Y/βL98W TCR enhanced the percentages of MART-1 homolog reactive T cells in seven homologs, but none reached statistical significance. However, it is difficult to discern the extent of how the supraphysiological TCR affinity affects the percentage of MART-1 and MART-1 homolog reactive T cells. Lastly, we observed the effect of the introduction of a MHC weakening TCR mutation on antigen specificity and cross-reactivity in TCR transduced CD8+ T cells. T cells expressing the αD26Y/αY50A/βL98W TCR demonstrated an overall trend in the reduction of the percentages of MART-1 homolog reactive CD8+ T cells compared to T cells expressing the WT TCR (0.25% - 4% vs. 1.4% - 18%), with statistical significance reached with HSV-1 glycoprotein III (2.3%) and M. tuberculosis protein I (2.3%). Albeit, these T cells also exhibited a statistically significant reduction in the percentage of MART-1 9mer reactive T cells compared to the WT TCR (29% vs. 44%). This indicated the addition of the αY50A mutation to the αD26Y/βL98W TCR was enough to reduce percentages of cross-reactive T cells, even below WT levels, but at the expense of T cell potency against MART-1 loaded targets. As observed with the CD4+ T cells, the introduction of the αY50V TCR mutation yielded different results than the introduction of the αY50A TCR mutation. Although not statistically significant, there was a trend towards a
reduction in the percentage of MART-1 9mer reactive T cells in T cells expressing the αD26Y/αY50V/βL98W TCR compared to T cells expressing the WT TCR. Furthermore, the pattern of reactivity against MART-1 homologs exhibited by T cells expressing the αD26Y/αY50V/βL98W TCR was similar to that of T cells expressing the WT TCR. In summary, these results indicated that the percentage of MART-1 homolog reactive T cells was enhanced in CD8⁺ T cells expressing the αD26Y TCR or αD26Y/βL98W TCR, indicating, cross-reactivity is augmented by CD8. Lastly, our structure-guided design strategy worked, as CD8⁺ T cells expressing the αD26Y/αY50A/βL98W TCR exhibited reduced cross-reactivity compared to T cells expressing the WT TCR. However, this was at the expense of reduced MART-1 specific T cell reactivity.

We previously described how the 3D TCR affinity correlated to the antigen specificity and cross-reactivity of CD4⁺ T cells transduced to express the WT TCR or each mutant DMF5 TCR. The 3D TCR affinity measurements did not take into account the CD8 co-receptor, however, it was of interest to determine if the presence of the CD8 co-receptor affected any of the previous correlations. The observed percentages of MART-1 and MART-1 homolog reactive TCR transduced T cells generally correlated to 3D affinity in the CD8⁺ T cells, with a few exceptions. Specifically, there was an overall increase in the percentage of MART-1 homolog reactive T cells expressing the αD26Y TCR compared to the WT TCR, coinciding with the increased in TCR affinity. However, despite the 3-fold TCR affinity enhancement in the βL98W TCR, the percentages of MART-1 and MART-1 homolog reactive T cells were generally similar to T cells expressing the WT TCR. The statistically significant reduction in the percentage of MART-1 reactive T cells in T cells expressing the αD26Y/βL98W TCR was most likely
again, attributed to its high affinity. Although not significant, trends in increased percentages of MART-1 homolog reactive αD26Y/βL98W TCR expressing T cells were observed compared to T cells expressing the WT TCR, coinciding with 3D TCR affinity. As predicted based on its lower 3D TCR affinity, T cells expressing the αD26Y/αY50A/βL98W TCR exhibited a significant reduction in the percentage of MART-1 reactive T cells compared to T cells expressing the WT TCR, and an overall reduction in MART-1 homolog reactive T cells, reaching statistical significance in two (HSV-1 glycoprotein I and M. tuberculosis protein I). Lastly, although not statistically significant, T cells expressing the αD26Y/αY50V/βL98W TCR displayed a trend toward a reduction in the percentage of MART-1 reactive T cells compared to T cells expressing the WT TCR, coinciding with 3D TCR affinity. However, the percentages of MART-1 homolog αD26Y/αY50V/βL98W TCR expressing reactive T cells were similar to T cells expressing the WT TCR. Overall, there were examples where 3D TCR affinity correlated to MART-1 reactivity and cross-reactivity in TCR transduced CD8+ T cells, but this was not the case with every DMF5 TCR. Specifically, TCR transduced CD8+ T cells harboring DMF5 TCRs with the highest or lowest 3D affinities compared to the WT TCR (αD26Y, αD26Y/βL98W, and αD26Y/αY50A/βL98W) generally correlated with MART-1 homolog reactivity and/or MART-1 reactivity.

Thus far we have observed how mutations in the DMF5 TCR affected recognition of the MART-1 homologs by comparing the average percentages of antigen reactive T cells amongst six experiments. These results demonstrated trends in how mutations in the DMF5 TCR affected recognition of individual MART-1 homologs. However, it was difficult to observe the effect of mutations in the DMF5 TCR on the overall, or total, level
of cross-reactivity compared the WT TCR. Therefore, we wanted to quantitatively examine how mutations in the DMF5 TCR affected the overall level of cross-reactivity compared to the WT TCR. For this analysis we focused only on CD8$^+$ T cells for a few reasons. First, compared to CD4$^+$ T cells expressing the WT TCR, only statistically significant differences in MART-1 homolog reactivity were observed in CD4$^+$ T cells expressing the αD26Y TCR or αD26Y/βL98W TCR (Figure 19). Secondly, only two out of the eleven MART-1 homologs elicited more than 1% WT TCR expressing reactive CD4$^+$ T cells (Figure 19). Furthermore, these two MART-1 homologs elicited lower than 2% WT TCR expressing reactive CD4$^+$ T cells (Figure 19). The purpose of this analysis was to determine how each modified DMF5 TCR altered total cross-reactivity compared to the WT TCR. Since CD8$^+$ T cells were much more cross-reactive, we focused solely on CD8$^+$ T cells for this analysis of cross-reactivity.

We compared the percentages of MART-1 homolog reactive CD8$^+$ T cells between each of the modified DMF5 TCRs and the WT DMF5 TCR on a per experiment basis (Figure 21). Each data point (X, Y value) represents the percentages of reactive CD8$^+$ T cells in response to a MART-1 homolog by both the WT DMF5 TCR (X-axis) and a modified DMF5 TCR (Y-axis) in the same experiment. Numbers in the upper left square of each graph (51, 31, 32, 20, and 28) indicate the total number of occurrences there was an increased frequency of reactive T cells against a MART-1 homolog by a modified DMF5 TCR, relative to the WT TCR in the six experiments. Conversely, the numbers in the bottom right square of each graph (5, 20, 22, 34, and 23) indicate the total number of occurrences there was an increased frequency of MART-1 homolog reactivity by the WT TCR, relative to a modified DMF5 TCR in the six experiments.
There are a total of 66 data points from the six experiments, however, values of 0, 0 were not counted. Therefore, the numbers in the top left and the bottom right corners of each graph serve as an index of the frequency of cross-reactivity. In the first graph, the percentages of MART-1 homolog reactive WT TCR expressing T cells were plotted against the percentages of MART-1 homolog reactive WT TCR expressing T cells. Thus, a linear regression is observed with a slope of 1 and a Y-intercept of 0. Overall, there are three pieces of information to analyze from these data. The first piece of information is the number of occurrences in which a modified TCR elicited a higher percentage of MART-1 homolog specific T cells compared to the WT TCR. This is a quantitative, yes/no measurement that does not take into account differences in magnitudes. The second piece of information is the slope of the linear regression line. This measurement takes into account the differences in magnitudes amongst a modified TCR and the WT TCR and overall indicates how the modified DMF5 TCR affected recognition of the MART-1 homologs in relation to the WT DMF5 TCR. Thirdly, the Y-intercept represents the average percentage of cross-reactive T cells expressing a modified TCR, when the percentage of cross-reactive WT TCR expressing T cells is 0. Specifically, the higher the Y-intercept, the more cross-reactive the modified TCR is. In the second graph, the percentages of MART-1 homolog reactive CD8+ T cells expressing the WT TCR is plotted against the percentages of MART-1 homolog reactive CD8+ T cells expressing the αD26Y TCR. CD8+ T cells expressing the αD26Y TCR dramatically enhanced overall cross-reactivity compared to T cells expressing the WT TCR, specifically, in 51/56 responses against the MART-1 homologs.
Figure 21. Impact of DMF5 TCR Mutations in TCR Transduced CD8+ T Cells against MART-1 Homologs. The individual percentages of MART-1 homolog reactive CD8+ T cells (from Figure 20) were plotted for each TCR with the percentage of reactive T cells expressing each modified TCR on the Y-axis and the percentage of reactive T cells expressing the WT TCR on the X-axis. Data from all 6 experiments (two independent repeats in three donors) are plotted to total 66 data points, each point being plotted from the same experiment. Numbers in the upper left square indicate the total number of occurrences there was an increased frequency of reactive T cells against a MART-1 homolog by a modified TCR relative to the WT TCR and conversely, the numbers in the bottom right square indicate the total number of occurrences there was an increased frequency of reactive T cells against a MART-1 homolog by the WT TCR relative to a modified TCR. Values of 0, 0 were not counted. Linear regression line and the equation are shown on each graph.
For simplicity, the frequency in which each modified DMF5 TCR resulted in an increase in the percentages of MART-1 homolog reactive CD8\(^+\) T cells relative to the WT TCR is shown in Table 7. Percentages over 50% indicated the modified DMF5 TCR, overall, exhibited an increase in the percentages of MART-1 homolog reactive CD8\(^+\) T cells compared to the WT TCR. Specifically, T cells expressing the αD26Y TCR demonstrated an increased percentage of MART-1 homolog reactive T cells in 91% (51/56) of responses compared to the WT TCR. Here, the denominator is 56, not 66, because values of 0, 0 were not counted. This conclusion was further supported by observing a slope above 1 (1.3633) in the calculated linear regression. The Y-intercept of 9.1601 indicates that on average, 9.2% of αD26Y TCR expressing T cells were cross-reactive when 0% of WT TCR expressing T cells were cross-reactive. Overall, these data quantitatively demonstrated the αD26Y TCR mutation elicited an increase in the level of cross-reactivity compared to the WT TCR. In the third graph, T cells expressing the βL98W TCR demonstrated an increased percentage of MART-1 homolog reactive CD8\(^+\) T cells in 60.8% (31/51) of responses compared to the WT TCR (Table 7). Interestingly, the slope of the linear regression line was less than 1 (0.6358), indicating there was an increase in the percentages of MART-1 homolog reactive WT TCR expressing T cells. This illustrates the difference in conclusions that can be made about cross-reactivity based upon the frequency calculation or the slope calculation. Specifically, the frequency is based upon a yes/no calculation, whereas the slope accounts for changes in magnitudes between the modified DMF5 TCRs and the WT DMF5 TCR in all 6 experiments.
Therefore, cross-reactivity is enhanced, or it is not enhanced, with CD8+ T cells expressing the βL98W TCR compared to the WT TCR, depending upon how the data is analyzed. The Y-intercept of 1.3796 indicates that on average, 1.4% of βL98W TCR expressing T cells were cross-reactive when 0% of WT TCR expressing T cells were cross-reactive. Overall, these data indicated in terms of the number of occurrences and the Y-intercept, the βL98W TCR mutation enhanced cross-reactivity compared to the WT TCR. However, in terms of the magnitudes of overall cross-reactivity, the βL98W TCR mutation decreased cross-reactivity compared to the WT TCR. In the fourth graph, CD8+ T cells expressing the αD26Y/βL98W TCR demonstrated an increased percentage of MART-1 homolog reactive T cells in 59.2% (32/54) of responses compared to the WT TCR (Table 7). Conversely, the slope of the linear regression line was less than 1 (0.534), indicating there was an increase in the percentages of MART-1 homolog reactive WT TCR expressing T cells. The Y-intercept of 3.5174 indicates that

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Table 7. Frequencies of Occurrences of Mutated DMF5 TCRs with Enhanced MART-1 Homolog Recognition Compared To the WT TCR.
on average, 3.5% of αD26Y/βL98W TCR expressing T cells were cross-reactive when 0% of WT TCR expressing T cells were cross-reactive. Overall, these data indicated in terms of the number of occurrences and the Y-intercept, the αD26Y/βL98W TCR mutation enhanced cross-reactivity compared to the WT TCR. However, in terms of the magnitudes of overall cross-reactivity, the αD26Y/βL98W TCR mutations decreased cross-reactivity compared to the WT TCR. In the fifth graph, CD8+ T cells expressing the αD26Y/αY50A/βL98W TCR demonstrated an increased percentage of MART-1 homolog reactive T cells in 37% (20/54) of responses compared to the WT TCR (Table 7). However, a majority of the cross-reactive values that were enhanced with the αD26Y/αY50A/βL98W TCR were very similar to the WT TCR values and were under 4%. The close proximity of some these values is therefore, unseen with this frequency quantification, but rather is more apparent in the stark decrease in the slope. Specifically, this result is further supported by the observation of the slope far below 1 (0.1356) in the calculated linear regression. The Y-intercept of 0.6643 indicates that on average, 0.67% of αD26Y/αY50A/βL98W TCR expressing T cells were cross-reactive when 0% of WT TCR expressing T cells were cross-reactive. Overall, these data quantitatively demonstrate the αD26Y/αY50A/βL98W TCR mutations elicited a decrease in the level of cross-reactivity compared to the WT TCR. Lastly, CD8+ T cells expressing αD26Y/αY50V/βL98W TCR demonstrated an increased percentage of MART-1 homolog reactive T cells in 54.9% (28/51) of responses compared to the WT TCR (Table 7). However, the slope of the linear regression line (0.7047) implicated when taking magnitudes into account, cross-reactivity was reduced with the αD26Y/αY50V/βL98W TCR compared to the WT TCR. The Y-intercept of 0.9032 that
on average, 0.9% of αD26Y/αY50V/βL98W TCR expressing T cells were cross-reactive when 0% of WT TCR expressing T cells were cross-reactive. Overall, these data indicated in terms of the number of occurrences, the αD26Y/αY50V/βL98W TCR mutations enhanced cross-reactivity compared to the WT TCR. However, in terms of the magnitudes of overall cross-reactivity, the αD26Y/αY50V/βL98W TCR mutation decreased cross-reactivity compared to the WT TCR.

In totality, these results exemplify the different conclusions that can be inferred about cross-reactivity based upon the occurrences of cross-reactivity or the magnitudes of cross-reactivity. It is plausible that both of these conclusions have biological implications for cross-reactivity in a human. Conclusively, these results quantitatively imply that overall cross-reactivity against MART-1 homologs was enhanced in CD8+ T cells expressing the αD26Y TCR and was decreased in CD8+ T cells expressing the αD26Y/αY50A/βL98W TCR compared to CD8+ T cells expressing the WT DMF5 TCR.

Thus far we have determined how mutations in the DMF5 TCR altered cross-reactivity in CD4+ and CD8+ T cells by observing recognition of sequence homologous MART-1 peptides. We have also observed how mutations in the DMF5 TCR affect cross-reactivity in totality, compared to the WT TCR. It would be beneficial to elucidate if there were any correlations between the structural aspects of the introduced DMF5 TCR mutations and changes observed in MART-1 homolog reactivity compared to the WT DMF5 TCR. To do this we individually compared each of the average percentages of MART-1 and MART-1 homolog reactive CD4+ and CD8+ T cells between each modified DMF5 TCR with the WT DMF5 TCR. Specifically, we listed the peptides in descending order based on the percent antigen reactive T cells to compare differences in the order
of recognition between the WT DMF5 TCR and a modified DMF5 TCR. For this analysis, recognition of a peptide by less than 2% of TCR transduced T cells and differences of less than 2% amongst two different TCRs, were not considered for comparative analysis. Since the percentages of antigen reactive T cells are the averages of six experiments, these thresholds allowed us to better compare overall trends in the altered recognition of the MART-1 homologs. Table 8 represents the percentages of reactive T cells expressing either the WT TCR or αD26Y TCR in CD4+ and CD8+ T cells. As previously described, the percent antigen reactive T cells expressed any one or more functional markers (CD107A or cytokines). In CD4+ T cells, T cells expressing the αD26Y TCR exhibited enhanced reactivity against every MART-1 homolog compared to T cells expressing the WT TCR. Consequently, it is difficult to make specific comparisons as T cells expressing the WT TCR hardly recognized the MART-1 homologs. Specifically, only two out of the eleven MART-1 homologs elicited reactivity in more than 1% of WT TCR expressing CD4+ T cells and these two MART-1 homologs elicited reactivity in less than 2% of WT TCR expressing CD4+ T cells. However, there are a few points to make in regards to the order of MART-1 homologs recognized by αD26Y TCR expressing CD4+ T cells. The most highly recognized MART-1 homolog maintained homology in the peptide core, HSV-1 glycoprotein III (GIGI). This hydrophobic “GIGI” at positions 3 through 6 has been shown to be important for DMF5 TCR recognition [404, 423]. Secondly, CD4+ T cells expressing the αD26Y TCR recognized the ADP-ribose diphosphatase peptide. This was most likely due to the removal of unfavorable charge repulsed with the WT DMF5 TCR and the aspartic acid at position two of the peptide.
### Table 8. Summary of Percentage of MART-1 Homolog Reactive T Cells Expressing the WT or αD26Y TCR.

The differences in sequence homology with the MART-1 9mer is in bold and underlined. The percent reactive T cells are the average of six independent experiments. The percent reactive T cells was determined following background subtraction, as described in Chapter Two. Antigen reactive T cells expressed any one or more functional marker.

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Lastly, this tyrosine TCR mutation at position 26 in the TCR alpha chain was proposed to have an effect on the N-terminal region of the peptide. However, there appears to be no apparent preference for N-terminal peptide residues. Specifically, the top four recognized MART-1 homologs each contain a different amino acid at position one. This indicated the tyrosine mutation non-specifically enhanced recognition of the MART-1 homologs. Overall, αD26Y TCR mutation in the DMF5 TCR non-specifically enhanced the percentages of CD4⁺ reactive T cells against all of the MART-1 homologs compared to T cells expressing the WT TCR.

We next examined how the presence of the CD8 co-receptor affected any structural correlations between the αD26Y TCR mutation and recognition of MART-1 homologs. In the CD8⁺ T cells, T cells expressing the αD26Y TCR again demonstrated an increase in all the percentages of MART-1 homolog reactive T cells compared to T cells expressing the WT TCR. One notable difference in recognition was with the GPCR 3 peptide, as it was not recognized by CD8⁺ T cells expressing the WT TCR. The stark increase in the percent αD26Y TCR expressing reactive CD8⁺ T cells could be due to enhanced binding with the N-terminal end of the peptide or an overall favorable antigenic surface due to the four mutations (positions 2, 4, 6, and 8) in the GPCR 3 peptide. Like in the CD4⁺ T cells, there appears to be no apparent preference for N-terminal peptide residues by CD8⁺ T cells expressing the αD26Y TCR. Overall, the αD26Y mutation non-specifically enhanced the percentages of MART-1 homolog reactive T cells compared to T cells expressing the WT TCR in both CD4⁺ and CD8⁺ T cells. Moreover, CD8 augmented the magnitudes of cross-reactivity.
We subsequently elucidated any structural correlations between MART-1 homolog recognition and the tryptophan mutation at position 98 in the TCR beta chain. Table 9 represents the percentage of antigen reactive T cells expressing either the WT TCR or βL98W TCR in TCR transduced CD4+ and CD8+ T cells. Contributions of the tryptophan substitution in the βL98W TCR are hard to elucidate in CD4+ T cells because as like CD4+ T cells expressing the WT TCR, they were not very reactive against the MART-1 homologs. Specifically, seven MART-1 homologs were recognized by less than 2% of βL98W TCR expressing CD4+ T cells. However, the percentage of βL98W TCR expressing ADP-ribose diphosphatase reactive CD4+ T cells was enhanced compared to CD4+ T cells expressing the WT TCR (5.9% vs. 1.67%). This could be attributed to the leucine at position 7 because the βL98W mutation enhanced shape complementarity with position 7 in the MART-1 9mer peptide. Albeit, this is not reproducible in other MART-1 homologs with that contain a leucine at position 7 (HSV-1 glycoprotein III, GPCR 3, β-endoxylanase). Overall, the tryptophan mutation at position 98 in the TCR beta chain did not preferentially enhance recognition of MART-1 homologs with a leucine at position 7.

We next examined how the presence of the CD8 co-receptor affected any structural correlations between the βL98W mutation and recognition of MART-1 homologs. The percentages and order of MART-1 homolog reactive CD8+ T cells is fairly similar between CD8+ T cells expressing the WT TCR or βL98W TCR. One notable difference was the increase in the percentage of human CD9 reactive T cells in T cells expressing the βL98W TCR compared to the WT TCR (9.43% vs. 3.02%).
Table 9. Summary of Percentage of MART-1 Homolog Reactive T Cells Expressing the WT or βL98W TCR. The differences in sequence homology with the MART-1 9mer is in bold and underlined. The percent reactive T cells are the average of six independent experiments. The percent reactive T cells was determined following background subtraction, as described in Chapter Two. Antigen reactive T cells expressed any one or more functional marker.

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Human CD9 contains an alanine at position 7, and thus, it is possible that the tryptophan in the βL98W TCR enhanced binding with the small nonpolar alanine. Overall, the tryptophan in the βL98W TCR did not significantly affect the pattern of recognition against the MART-1 homologs compared to T cells expressing the WT TCR. Furthermore, the tryptophan mutation at position 98 in the TCR beta chain did not preferentially enhance recognition of MART-1 homologs with a leucine at position 7 in CD4+ or CD8+ T cells.

We next elucidated any structural correlations between MART-1 homolog recognition and the combination of the αD26Y TCR and βL98W TCR mutations. Table 10 represents the percentage of antigen reactive T cells expressing either the WT TCR or αD26Y/βL98W TCR in CD4+ and CD8+ T cells. In CD4+ T cells, the αD26Y/βL98W TCR generally enhanced the percentage of MART-1 homolog reactive T cells compared to T cells expressing the WT TCR. In αD26Y/βL98W TCR expressing CD4+ T cells, the MART-1 homologs that elicited the highest percentages of reactive T cells were similar to T cells expressing the αD26Y TCR (M. tuberculosis protein I, HSV-1 glycoprotein III, ADP-ribose diphosphatase, human CD9, HREEP 5). These results implicated the structural effect of the αD26Y TCR mutation. Surprisingly, 6.8% of αD26Y TCR expressing CD4+ T cells recognized the LmrA MART-1 homolog, while only 1.23% of αD26Y/βL98W TCR expressing CD4+ T cells recognized LmrA. However, as discussed previously, it is possible that the affinity of the αD26Y/βL98W TCR is too high and thus, T cell function has reached its maximum threshold, or has been arrested. Moreover, 3D αD26Y/βL98W TCR affinity measurements with the MART-1 10mer indicated a nanomolar value.
Table 10. Summary of Percentage of MART-1 Homolog Reactive T Cells Expressing the WT or αD26Y/βL98W TCR. The differences in sequence homology with the MART-1 9mer is in bold and underlined. The percent reactive T cells are the average of six independent experiments. The percent reactive T cells was determined following background subtraction, as described in Chapter Two. Antigen reactive T cells expressed any one or more functional marker.

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This indicated anchor residues could very much affect 3D TCR affinity. Therefore, it is difficult to correlate αD26Y/βL98W TCR structure with T cell responses against the MART-1 homologs if the T cell functional output has been subsequently affected due to the supraphysiological high affinity. Patterns of the percentages of MART-1 homolog reactive αD26Y/βL98W TCR expressing CD8+ T cells generally coincided with the hypothesis associated with TCR affinity being too high and subsequently arresting T cell function. For example, 7.73% of αD26Y TCR expressing CD8+ T cells recognized the HREEP 5 peptide, while only 1.2% αD26Y/βL98W TCR expressing CD8+ T cells recognized the HREEP 5 peptide. However, we would expect a higher percentage of reactive T cells with higher affinity TCR. Furthermore, CD4+ and CD8+ αD26Y/βL98W TCR expressing T cells recognized ADP-ribose diphosphatase with the same percentage of T cells (5.2% vs. 5.25%). A similar trend was observed in CD4+ T cells vs CD8+ T cells with HSV-1 glycoprotein III (10.18% vs. 13.33%) and M. tuberculosis protein I (10.57% vs 10.68%) peptides. These results indicated the addition of CD8 had no effect on the percentage of reactive T cells. Overall, these results suggested specific patterns of MART-1 homolog recognition observed in αD26Y/βL98W TCR expressing CD4+ and CD8+ T cells and how they structurally correlate with the TCR mutations are difficult to elucidate because the supraphysiological affinity is most likely affecting T cell function.

We next elucidated any structural correlations between MART-1 homolog recognition and the introduction of a MHC weakening TCR mutation, αY50A, with the αD26Y and βL98W TCR mutations. Table 11 represents the percentage of antigen reactive T cells expressing either the WT TCR or αD26Y/αY50A/βL98W TCR in CD4+
and CD8+ T cells. Structural contributions of the αD26Y/αY50A/βL98W TCR mutations are hard to make in CD4+ T cells, because as observed with T cells expressing the WT TCR, they are minimally reactive against the MART-1 homologs. Specifically, only one MART-1 homolog (HREEP 5) was recognized by over 2% of αD26Y/αY50A/βL98W TCR expressing CD4+ T cells, at 2.13%. Overall, the introduction of the αD26Y/αY50A/βL98W TCR mutations did not affect MART-1 homolog recognition in CD4+ T cells compared to the WT TCR.

In αD26Y/αY50A/βL98W TCR expressing CD8+ T cells, only a small percentage of MART-1 homolog reactive T cells were observed. The three peptides recognized by over 2% of αD26Y/αY50A/βL98W TCR expressing CD8+ T cells were the same top three recognized by CD8+ T cells expressing the WT TCR (ADP-ribose diphosphatase, M. tuberculosis protein I, HSV-1 glycoprotein III). These peptides reveal sequence homology with the MART-1 9mer at positions 3 through 6. In summary, T cells expressing the αD26Y/αY50A/βL98W TCR exhibited a reduction in the percentages of MART-1 homolog reactive T cells, indicating the αY50A TCR mutation counterbalanced the αD26Y and βL98W TCR mutations. Moreover, the structure of the αD26Y/αY50A/βL98W TCR had no impact on the patterns of MART-1 homolog recognition compared to the WT TCR.

We next elucidated any structural correlations between MART-1 homolog recognition and the introduction of a different MHC weakening TCR mutation, αY50V, with the αD26Y TCR and βL98W TCR mutations. Table 12 represents the percentage of antigen reactive T cells expressing either the WT TCR or αD26Y/αY50V/βL98W TCR in CD4+ and CD8+ T cells.
Table 11. Summary of Percentage of MART-1 Homolog Reactive T Cells Expressing the WT or αD26Y/αY50A/βL98W TCR. The differences in sequence homology with the MART-1 9mer is in bold and underlined. The percent reactive T cells are the average of six independent experiments. The percent reactive T cells was determined following background subtraction, as described in Chapter Two. Antigen reactive T cells expressed any one or more functional marker.

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Table 12. Summary of Percentage of MART-1 Homolog Reactive T Cells Expressing the WT or αD26Y/αY50V/βL98W TCR. The differences in sequence homology with the MART-1 9mer is in bold and underlined. The percent reactive T cells are the average of six independent experiments. The percent reactive T cells was determined following background subtraction, as described in Chapter Two. Antigen reactive T cells expressed any one or more functional marker.
Contributions of the αD26Y/αY50V/βL98W TCR mutations are hard to make in CD4+ T cells, as with T cells expressing the WT TCR, they are minimally reactive against the MART-1 homologs. However, HREEP 5 and ADP-ribose diphosphatase elicited the highest percentages of αD26Y/αY50V/βL98W CD4+ reactive T cells (4.88% and 4.07%). The percentage of HREEP 5 reactive CD4+ T cells was similar between αD26Y/αY50V/βL98W TCR and αD26Y/βL98W TCR expressing CD4+ T cells (4.88% vs. 4.07%), but less than αD26Y TCR expressing T cells (9.07%). This again suggested the αD26Y/βL98W TCR affinity was too high, thus attenuating T cell potency, but the addition of the αY50V mutation could offset the effect of the other MART-1 targeting mutations. It was interesting that the MART-1 homolog, HREEP 5, elicited the highest percentages of reactive CD4+ T cells with the αD26Y/αY50A/βL98W TCR and αD26Y/αY50V/βL98W TCR, but none of the other TCRs. HREEP 5 only shares sequence homolog with the MART-1 9mer at positions 4 and 5. These results implicate that the addition of a TCR mutation that eliminates or weakens binding with three residues in the MHC to the αD26Y/βL98W TCR, could have less of an effect on recognition of HREEP 5 compared to the other MART-1 homologs. This could be due to the structural, antigenic surface of HREEP 5 in the MHC. In summary, the introduction of the αD26Y/αY50V/βL98W TCR mutations had minimal overall effects on the patterns in recognition of the MART-1 homologs compared to the WT TCR in CD4+ T cells.

We next examined how the presence of the CD8 co-receptor affected any structural correlations between the αD26Y/αY50V/βL98W TCR mutations and recognition of MART-1 homologs. In the αD26Y/αY50V/βL98W TCR expressing CD8+ T cells, the order of MART-1 homolog recognition was the same, for MART-1 homologs
that elicited reactivity, compared to T cells expressing the WT TCR. In summary, these results indicated that these three TCR mutations did not significantly affect structural recognition of the MART-1 homologs compared to T cells expressing the WT TCR.

These results of this analysis highlight a few key points in regards to TCR structure and antigen recognition. First, CD8 generally augments antigen recognition. Secondly, recognition of MART-1 homologs did not always align with predictions based upon the structural impact of the introduced DMF5 TCR mutations. Specifically, the αD26Y TCR mutation non-specifically enhanced all MART-1 homolog recognition and the βL98W TCR mutation did not specifically enhance recognition of MART-1 homologs with a leucine at position 7. Conclusively, this analysis indicated the net antigenic surface of the whole pMHC complex is more important than individual or specific alterations in peptide residues.

**Impact of Mutant DMF5 TCRs on Polyfunctional T cell Phenotypes**

Our conclusions about cross-reactivity thus far have been based solely on reactive T cells determined by expression of any one or more functional markers (CD107A, IFN-γ, TNF-α, IL-17A, IL-2, IL-4, or IL-22). However, it has been shown that polyfunctional phenotypes are important in different effector T cell functions. Therefore, we sought to determine how mutations in the DMF5 TCRs altered polyfunctional phenotypes of TCR transduced T cells. Since seven functional markers were evaluated (CD107A, IFN-γ, TNF-α, IL-17A, IL-2, IL-4, and IL-22), there was a potential for each T cell to exhibit 1 of 128 (2^7) possible functional phenotypes. “Cool plots” were generated to display how mutations in the DMF5 TCR altered functional phenotypes compared to the WT DMF5 TCR in different donors. We first focused on the CD4+ T cells to
determine how the structure of the TCR/pMHC interaction affected polyfunctional T cell responses. A representative example of the CD4+ reactivity against T2 cells loaded with the MART-1 9mer is shown in Figure 22. The percentage of reactive CD4+ T cells expressing a specific functional phenotype ranged from 0% to about 6.5%. As described in Chapter Two, reactivity is defined after background subtraction (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). Specifically, any phenotype expressed by over 0.1% of TCR transduced T cells were defined as reactive. Previous studies performing this analysis focused on phenotypes elicited by over 0.25% of TCR transduced T cells [95]. Thus, we decided to be more conservative by examining phenotypes elicited by over 0.1% of any DMF5 TCR transduced T cell population. Here, among six TCR transduced CD4+ T cell populations, 25 out of the potential 128 polyfunctional phenotypes were detected in at least one DMF5 TCR transduced T cell population, by over 0.1% of TCR transduced CD4+ T cells. It is evident that individual TCR transduced CD4+ T cells are very polyfunctional and are capable of producing Th1 (IFN-γ, TNF-α, IL-2), Th2 (IL-4), and Th17 (IL-17A and IL-22) cytokines [424, 425]. Th1, Th2, and Th17 cytokine producing TCR transduced CD4+ T cells are also capable of lysis, as measured by CD107A. Furthermore, the production of Th1, Th2, and Th17 cytokines are not mutually exclusive, meaning TCR transduced T cells produced combinations of cytokines from the different T cell subsets. Overall, these results indicated TCR transduced CD4+ T cells were capable of exhibiting functional phenotypes comprised of lysis and Th1, Th2, and Th17 cytokines.
We subsequently observed how the distribution of functional phenotypes was altered amongst T cells expressing each of the different DMF5 TCRs when stimulated with the MART-1 9mer. It is evident that there is variability in the CD107A and cytokine responses amongst the different DMF5 TCRs even when presented with the same ligand in the same donor and the same experiment (Figure 22). For example, the first listed polyfunctional phenotype consisted of CD107A⁺, IFN-γ⁺, TNF-α⁺, IL-4⁺ TCR transduced CD4⁺ T cells. This phenotype consisted of both type 1 and type 2 cytokines and lysis. This phenotype was exhibited in less than 0.5% of αD26Y TCR, βL98W TCR, and αD26Y/αY50V/βL98W TCR expressing T cells but absent in T cells expressing the other DMF5 TCRs. In a second example, the second functional phenotype consisted of CD107A⁺, IFN-γ⁺, TNF-α⁺ TCR transduced CD4⁺ T cells. This phenotype was present in about 2% to 3% of T cells expressing the WT TCR, βL98W TCR, and αD26Y/αY50V/βL98W TCR, less than 0.5% of T cells expressing the αD26Y TCR, and was not exhibited in T cells expressing the αD26Y/βL98W TCR or αD26Y/αY50A/βL98W TCR. Another example of this variability between the different DMF5 TCRs was observed in the TNF-α⁺ only phenotype. This phenotype was present in around 1% to 2% of T cells expressing the WT TCR, αD26Y TCR, and αD26Y/αY50V/βL98W TCR, less than 0.5% of T cells expressing the αD26Y/αY50A/βL98W TCR, and was not present in T cells expressing the αD26Y/βL98W TCR or βL98W TCR. Overall, these are a few of many examples of how the polyfunctional phenotypes expressed are TCR dependent in the same donor and experiment when presented with the same ligand.
Figure 22. Impact of DMF5 Mutations in TCR Transduced CD4+ T cells on Polyfunctional Responses against MART-1 (Donor One). The data indicate reactivity against T2 cells loaded with the MART-1 9mer peptide in donor one, experiment one. “Cool plots” were generated to include any functional phenotype present over 0.1%. In the functional phenotypes listed, “+” indicates positive for the marker and “-” indicates negative for the marker. As described in Chapter Two, reactivity is defined after background subtraction and specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). The color depicted in the “cool plot” correlates to the scale on the right. An “X” indicates no T cells were positive (≤0.1% after background subtraction) for given functional phenotype. The color white for a given functional phenotype indicates a percentage less than 0.5%.

Despite the variability in the magnitudes of responses and in the polyfunctional phenotypes amongst T cells expressing each of the different DMF5 TCRs, there are two functional phenotypes reproducibly exhibited with T cells expressing each of the DMF5 TCRs when stimulated with the MART-1 9mer (Figure 22). These two phenotypes are IFN-γ+, TNF-α+ and TNF-α+, IL-4+. TNF-α is type 1 cytokine while IL-4 is type 2 cytokine, thus exemplifying the ability for the T cells to commonly express cytokines that have been previously categorized into different T cell subsets. Overall, this experiment demonstrated the polyfunctionality of TCR transduced CD4+ T cells and the variability in
the functional phenotypes demonstrated in T cells expressing different DMF5 TCRs despite presentation of the same ligand. Despite variability in the patterns of polyfunctional phenotypes, two phenotypes were reproducibly exhibited by T cells expressing each of the DMF5 TCRs in response to the MART-1 9mer.

It is well appreciated that polyclonal PBL-derived T cells will not function in the exact same manner when stimulated on different days after previous activations. Specifically, the duration of in vitro cell culture after T cell activation can affect the expansion of different T cell populations [426]. Furthermore, one study indicated four day anti-CD3 T cell activation only induced proliferation of 60% of T cells, depending upon T cell populations [427]. It has also been shown that T cells will become less functional, the longer they are cultured in vitro [428]. Consequently, proportions of T cell populations and overall T cell function could vary depending upon the duration of in vitro cell culture. Therefore, we determined if the patterns described above were reproducible in a second repeat in donor one, with the same TCR transduced T cell culture, four days later (Figure 46, in the appendix). In the repeat of donor one among six TCR transduced CD4+ T cell populations, 31 out of the potential 128 polyfunctional phenotypes were detected in at least one DMF5 TCR transduced T cell population, by over 0.1% of TCR transduced CD4+ T cells. Six more phenotypes were represented in the second experiment, but the percentage of reactive CD4+ T cells expressing a specific functional phenotype decreased from a maximum of 6.5% to a maximum of 2.5%. These findings indicated the polyfunctional patterns are not completely reproducible in this donor. For example, the IFN-γ+, TNF-α+ and TNF-α+, IL-4+ phenotypes were exhibited by T cells expressing each DMF5 TCR in the first experiment. In the second experiment, the IFN-
γ+, TNF-α+ phenotype was exhibited by T cells expressing each DMF5 TCR. However, the second phenotype exhibited by T cells expressing each DMF5 TCR was CD107A+, IL-4+, rather than the TNF-α+, IL-4+ phenotype. In summary, these two repeats indicated that functional phenotypes are not completely reproducible in the same donor when observed in two independent experiments. However, one functional phenotype was reproducibly exhibited by CD4+ T cells expressing each of the DMF5 TCRs in both experiments in response to the MART-1 9mer.

Donor variability occurs in human polyclonal PBL-derived T cells. This phenomenon has been observed both in vitro and in vivo. Therefore, we performed this analysis in another donor to determine how mutations in the DMF5 TCR altered polyfunctional phenotypes in polyclonal PBL-derived T cells from a different donor when stimulated with the MART-1 9mer. A representative cool plot for the second donor is shown in Figure 23. In this experiment, the percentage of reactive CD4+ T cells expressing a specific functional phenotype ranged from 0% to around 9%. Among six TCR transduced CD4+ T cell populations, 71 out of the potential 128 polyfunctional phenotypes were detected in at least one DMF5 TCR transduced population, by over 0.1% of TCR transduced CD4+ T cells. It is evident that the TCR transduced T cells are again, very polyfunctional. Furthermore, phenotypes consisting of lysis and combinations of Th1, Th2 and Th17 cytokines were exhibited. Overall, these results indicated that this donor and experiment exhibited more functional phenotypes than donor one, and included functional phenotypes consisting of combinations of lysis and Th1, Th2 and Th17 cytokines.
Next, we examined how the distribution of functional phenotypes changed amongst T cells expressing each of the different DMF5 TCRs when stimulated with the MART-1 9mer. As observed with donor one, different polyfunctional phenotypes were exhibited by T cells expressing the different DMF5 TCRs despite presentation of the same ligand (Figure 23). For example, the first functional phenotype listed consisted of CD107A⁺, IFN-γ⁺, TNF-α⁺, IL-22⁺, IL-17A⁺, IL-2⁺ TCR transduced CD4⁺ T cells. This phenotype is exhibited in less than 0.5% of αD26Y TCR expressing T cells but absent in T cells expressing each of the other DMF5 TCRs. In another example, the last phenotype in the first row consisted of CD107A⁺ TCR transduced CD4⁺ T cells. This phenotype is exhibited in 2% of WT TCR and αD26Y/βL98W TCR expressing T cells, but absent in T cells expressing each of the other DMF5 TCRs. Overall, these are a few of the many examples of how the polyfunctional phenotypes expressed are TCR dependent in the same donor and experiment despite presentation of the same ligand. Despite the variability in polyfunctional phenotypes exhibited amongst T cells expressing each of the different DMF5 TCRs, there are 14 functional phenotypes that are reproducibly exhibited by T cells expressing each DMF5 TCR when stimulated with the MART-1 9mer (Figure 23). This observation indicated this donor, in this experiment, exhibited functional phenotypes consisting of lysis and cytokines from combinations of different T cell subsets. Overall, this experiment demonstrated the polyfunctionality of TCR transduced CD4⁺ T cells and the variability in the functional phenotypes demonstrated in T cells expressing each of the different DMF5 TCRs despite presentation of the same ligand. Nonetheless, 14 phenotypes were reproducibly exhibited by T cells expressing each of the DMF5 TCRs in this experiment.
Figure 23. Impact of DMF5 Mutations in TCR Transduced CD4\(^+\) T cells on Polyfunctional Responses against MART-1 (Donor Two). The data indicate reactivity against T2 cells loaded with the MART-1 9mer peptide in donor two, experiment one. “Cool plots” were generated to include any functional phenotype present over 0.1%. In the functional phenotypes listed, “+” indicates positive for the marker and “-” indicates negative for the marker. As described in Chapter Two, reactivity is defined after background subtraction and specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). The color depicted in the “cool plot” correlates to the scale on the right. An “X” indicates no T cells were positive (<0.1% after background subtraction) for given functional phenotype. The color white for a given functional phenotype indicates a percentage less than 0.5%.

<table>
<thead>
<tr>
<th>DMF5 TCR</th>
<th>αD26Y/yY50V/βL96W</th>
<th>αD26Y/yY50A/βL96W</th>
<th>αD26Y/βL96W</th>
<th>αD26Y/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD107A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IFNγ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TNFα</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-22</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>IL-17A</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>IL-4</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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</table>

We observed variability and similarity within the patterns of polyfunctionality when we compared two independent repeats in the first donor. Therefore, we determined if the patterns described above were reproducible in a second repeat in
donor two, with the same TCR transduced T cell culture, four days later (Figure 47, in the appendix). In the repeat of donor two among six TCR transduced CD4+ T cell populations, 67 out of the potential 128 polyfunctional phenotypes were detected in at least one DMF5 TCR transduced T cell population, by over 0.1% of TCR transduced CD4+ T cells. Four fewer phenotypes were represented in the second experiment and the percentage of reactive CD4+ T cells expressing a specific functional phenotype decreased from a maximum of 9% to a maximum of 7%. This indicated the patterns were not completely reproducible in this donor. For example, the IL-4+ only phenotype is exhibited by T cells expressing each DMF5 TCR in the second experiment, but only by T cells expressing the αD26Y TCR in the first experiment. However, nine functional phenotypes were reproducibly exhibited by T cells expressing each of DMF5 TCRs in the two experiments. These functional phenotypes included 1.) CD107A+, IFN-γ+, TNF-α+, 2.) CD107A+, IL-17A+, 3.) IFN-γ+, TNF-α+, IL-17+, 4.) IFN-γ+, TNF-α+, 5.) IFN-γ+, 6.) TNF-α+, IL-22+, IL-17A+, 7.) TNF-α+, IL-17A+, 8.) TNF-α+, and 9.) IL-17A+. These results indicated that in this donor, T cells expressing each of the DMF5 TCRs exhibited phenotypes consisting of combinations of lysis and Th1 and Th17 cytokines. In summary, these two repeats indicated that functional phenotypes are not completely reproducible in the same donor when observed in two independent repeats. However, nine functional phenotypes were reproducibly exhibited by T cells expressing each of the DMF5 TCRs in response to the MART-1 9mer in both experiments.

It would be important to observe the differences and similarities in patterns of polyfunctional phenotypes amongst the first two donors. However, to first determine if variability was reproducibly observed in more than two donors, we performed this
analysis in a third donor. A representative cool plot for the third donor is shown in Figure 24. In this donor, the percentage of antigen reactive CD4$^+$ T cells expressing a specific functional phenotype ranged from 0% to about 12%. Among six TCR transduced CD4$^+$ T cell populations, 44 out of the potential 128 polyfunctional phenotypes were detected in at least one DMF5 TCR transduced T cell population, by over 0.1% of TCR transduced CD4$^+$ T cells. It is evident that the TCR transduced T cells are again, very polyfunctional. Overall, these results indicated in this donor and experiment, TCR transduced T cells exhibited functional phenotypes comprised of lysis and Th$^1$, Th$^2$, and Th$^{17}$ cytokines.

We next examined how the distribution of functional phenotypes changed amongst T cells expressing each of the different DMF5 TCRs when stimulated with the MART-1 9mer. As with donors one and two, different polyfunctional phenotypes were exhibited by T cells expressing the different DMF5 TCRs despite presentation of the same ligand (Figure 24). For example, the first functional phenotype listed consisted of CD107A$^+$, IFN-$\gamma^+$, TNF-$\alpha^+$, IL-17A$^+$, IL-2$^+$ TCR transduced CD4$^+$ T cells. This phenotype was exhibited by less than 0.5% of $\alpha$D26Y/$\beta$L98W TCR expressing T cells but absent in T cells expressing each of the other DMF5 TCRs. In another example, the second functional phenotype listed consisted of CD107A$^+$, IFN-$\gamma^+$, TNF-$\alpha^+$, IL-2$^+$ TCR transduced CD4$^+$ T cells. This phenotype was expressed by less than 0.5% of $\alpha$D26Y/$\alpha$Y50V/$\beta$L98W TCR expressing T cells, but absent in T cells expressing each of the other DMF5 TCRs. If we move further down the cool plot to the CD107A$^+$, IL-4$^+$ phenotype, this phenotype is expressed by less than 0.5% of $\alpha$D26Y/$\alpha$Y50V/$\beta$L98W TCR expressing T cells, 6% of $\alpha$D26Y TCR expressing T cells, about 2.5% of $\beta$L98W
TCR and αD26Y/βL98W TCR expressing T cells, and absent in WT TCR and αD26Y/αY50A/βL98W TCR expressing T cells. Overall, these results demonstrated that the exhibited polyfunctional phenotypes are TCR dependent within the same donor and the same experiment despite presentation of the same ligand.

Despite the variability in polyfunctional phenotypes amongst T cells expressing each of the different DMF5 TCRs, there were seven functional phenotypes that were reproducibly exhibited by T cells expressing each of the DMF5 TCRs (Figure 24). These functional phenotypes included 1.) CD107A⁺, IFN-γ⁺, TNF-α⁺, 2.) CD107A⁺, IFN-γ⁺, 3.) IFN-γ⁺, TNF-α⁺, IL-2⁺, 4.) IFN-γ⁺, TNF-α⁺, IL-4⁺, 5.) IFN-γ⁺, TNF-α⁺, 6.) IFN-γ⁺, and 7.) TNF-α⁺, IL-2⁺. These results implicated that Th₁ and Th₂ cytokines were commonly expressed amongst in T cells expressing each different DMF5 TCR. Furthermore, combinations of lysis, Th₁, or Th₂ cytokines were sometimes expressed in the same phenotype. Overall, this experiment demonstrated the polyfunctionality of TCR transduced CD4⁺ T cells and the variability of the functional phenotypes demonstrated in T cells expressing different DMF5 TCRs despite presentation of the same ligand. Nonetheless, seven phenotypes were reproducibly exhibited by T cells expressing each of the DMF5 TCRs in response to the MART-1 9mer in this experiment.

We observed variability and similarity within the patterns of polyfunctionality when comparing two independent repeats in the first two donors. Therefore, we determined if the patterns described above were reproducible in a second repeat in the third donor, with the same TCR transduced T cell culture, four days later (Figure 48, in the appendix).
Figure 24. Impact of DMF5 Mutations in TCR Transduced CD4+ T cells on Polyfunctional Responses against MART-1 (Donor Three). The data indicate reactivity against T2 cells loaded with the MART-1 9mer peptide in donor three, experiment one. “Cool plots” were generated to include any functional phenotype present over 0.1%. In the functional phenotypes listed, “+” indicates positive for the marker and “-” indicates negative for the marker. As described in Chapter Two, reactivity is defined after background subtraction, specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). The color depicted in the “cool plot” correlates to the scale on the right. An “X” indicates no T cells were positive (<0.1% after background subtraction) for given functional phenotype. The color white for a given functional phenotype indicates a percentage less than 0.5%.
In the repeat of donor three among six TCR transduced T cell populations, 27 out of the potential 128 polyfunctional phenotypes were detected in over 0.1% of TCR transduced CD4+ T cells in this donor and this experiment. Seventeen fewer phenotypes were exhibited in the second experiment compared to the first experiment, indicating the patterns were not completely reproducible in this donor. For example, the TNF-α+ only phenotype was exhibited by T cells expressing the αD26Y TCR in the second experiment, but not in the first experiment. However, four functional phenotypes were reproducibly exhibited by T cells expressing each of DMF5 TCRs in the two experiments. These exhibited phenotypes included 1.) CD107A+, IFN-γ+, 2.) IFN-γ+, TNF-α+, 3.) IFN-γ+, and 4.) TNF-α+, IL-2+. These results indicated that in this donor, T cells expressing each of the DMF5 TCRs exhibited phenotypes consisting of lysis and/or Th1 and Th2 cytokines in response to the MART-1 9mer. In summary, these two repeats indicated that functional phenotypes are not completely reproducible in the same donor when observed in two independent repeats. However, four functional phenotypes were reproducibly exhibited by T cells expressing each of the DMF5 TCRs in both experiments.

Thus far, we have demonstrated that TCR transduced CD4+ T cells were lytic and polyfunctional. Furthermore, in CD4+ T cells there is no CD8 co-receptor and thus, interactions are dependent upon the TCR/pMHC. There are a few overarching conclusions that can be made based upon the polyfunctional responses of T cells expressing each of the DMF5 TCRs against the MART-1 9mer in different experiments and donors. The first set of conclusions encompassed variability and similarity amongst polyfunctional patterns between donors and experiments. Table 13 depicts a numerical
summary of the number of functional phenotypes observed and their reproducibility among each experiment and donor. First, the number of polyfunctional patterns exhibited was donor and experiment dependent. For example, donor two was more polyfunctional than donors one and three. Furthermore, no two experimental repeats exhibited the same number of functional phenotypes. Secondly, the number of reproducible patterns of polyfunctional phenotypes amongst T cells expressing each of the DMF5 T cells was experiment dependent. For example, in donor one, experiment one, one reproducible functional phenotype was exhibited with T cells expressing each of the DMF5 TCRs, whereas in experiment two, two reproducible functional phenotypes were exhibited. Thirdly, the number of reproducible patterns of functional phenotypes exhibited in T cells expressing each of the DMF5 TCRs was donor dependent. For example, the number of unique reproducible functional phenotypes in T cells expressing each of the DMF5 TCRs was different in every donor (donor 1 – one, donor 2 – nine, donor 3 – four). Fourthly, one functional phenotype was exhibited by CD4$^+$ T cells expressing each of the DMF5 TCRs in every donor and in every experiment. This was a Th$_1$ cytokine pattern, IFN-$\gamma^+$, TNF-$\alpha^+$. This makes sense as expression of these two cytokines can be mediated by the same transcription factors (STAT1 and NF-$\kappa$B) [424, 429-432]. Furthermore, IFN-$\gamma$ is considered an early response cytokine and shown to be important to modulate the adaptive immune response [433-435]. In summary, these data indicated polyfunctional responses of CD4$^+$ T cells are dependent upon the TCR, experiment, and donor. However, we demonstrated that in response to the MART-1 9mer, T cells expressing each of the DMF5 TCRs always exhibited the IFN-$\gamma^+$, TNF-$\alpha^+$ phenotype.
### Table 13. Summary of Polyfunctional Phenotypes in TCR Transduced CD4+ T cells amongst Six Experiments.

*Reproducible phenotypes with every DMF5 TCR indicate that within a specific experiment, all six TCR transduced CD4+ T cell populations (WT DMF5 TCR and each mutant DMF5 TCR) exhibited the functional phenotype.**Reproducible phenotypes with every DMF5 TCR within donor indicate that within a specific donor, all six TCR transduced CD4+ T cell populations (WT DMF5 TCR and each mutant DMF5 TCR) exhibited the functional phenotype in both experimental repeats.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Experiment</th>
<th>Total # of phenotypes exhibited</th>
<th># of phenotypes exhibited with every DMF5 TCR*</th>
<th># of reproducible phenotypes with every DMF5 TCR within donor**</th>
<th>reproducible phenotypes within CD4+ T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>25</td>
<td>1</td>
<td>1</td>
<td>IFN-γ*, TNF-α*</td>
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<td></td>
<td>2</td>
<td>27</td>
<td>5</td>
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</table>

The second set of conclusions encompassed variability and similarity amongst polyfunctional patterns between donors and experiments in regards to each of the DMF5 TCRs. Table 14 depicts a numerical summary of the number of functional phenotypes observed amongst T cells expressing each of the DMF5 TCRs in each experiment and donor. First, the number of polyfunctional phenotypes exhibited is DMF5 TCR dependent. For example, in donor one and experiment one, T cells expressing each of the DMF5 TCRs exhibited a different number of polyfunctional phenotypes. Secondly, the number of polyfunctional phenotypes exhibited amongst T cells expressing any given DMF5 TCR is experiment and donor dependent. For example, T cells expressing the WT DMF5 TCR exhibited a different number of polyfunctional phenotypes in five out of the six experiments. Thirdly, the total number and the average number of functional phenotypes amongst the six experiments,
generally trends towards a correlation between number of polyfunctional phenotypes and the overall percentages of antigen reactive TCR transduced CD4\(^{+}\) T cells. For example, in Figure 19, CD4\(^{+}\) T cells expressing the αD26Y TCR and the βL98W TCR exhibited a trend towards an increase in the percentage of MART-1 9mer reactive T cells, compared to T cells expressing the WT TCR, in the six experiments. Herein, CD4\(^{+}\) T cells expressing the αD26Y TCR and the βL98W TCR exhibited a trend towards an increase in the average and total number of functional phenotypes exhibited in the six experiments, compared to T cells expressing the WT TCR. Conversely, in Figure 19, CD4\(^{+}\) T cells expressing the αD26Y/βL98W TCR and the αD26Y/αY50A/βL98W TCR exhibited a trend towards (statistically significant in T cells expressing the αD26Y/αY50A/βL98W TCR) a reduction in the percentage of MART-1 9mer reactive T cells, compared to T cells expressing the WT TCR, in the six experiments. Herein, CD4\(^{+}\) T cells expressing the αD26Y/βL98W TCR and the αD26Y/αY50A/βL98W TCR exhibited a trend towards a reduction in the average and total number of functional phenotypes exhibited in the six experiments, compared to T cells expressing the WT TCR. The one exception to this correlation is T cells expressing the αD26Y/αY50V/βL98W TCR exhibited a comparable percentage of MART-1 9mer reactive T cells but exhibited an increase in the average and total number of functional phenotypes, compared to T cells expressing the WT TCR. Conclusively, these results indicated polyfunctional phenotypes exhibited amongst T cells expressing each of the DMF5 TCRs is TCR, experiment, and donor dependent. However, CD4\(^{+}\) T cells expressing each of the DMF5 TCRs always exhibited the IFN-γ\(^{+}\), TNF-α\(^{+}\) functional phenotype in response to the MART-1 9mer.
Lastly, the number of different functional phenotypes exhibited generally correlated to the overall pattern in the percentages of MART-1 9mer reactive TCR transduced CD4+ T cells. Specifically, the greater the percentage of antigen reactive TCR transduced T cells, the more functional phenotypes exhibited.

The analysis of TCR transduced CD4+ T cells allowed us to examine how the structure of the TCR/pMHC altered polyfunctional T cell responses. However, as mentioned previously, CD8 has been shown to affect antigen recognition and T cell function. Therefore, we next examined how mutations in the DMF5 TCR altered the polyfunctional responses of CD8+ T cells. A representative example of the CD8+ reactivity against T2 cells loaded with the MART-1 9mer is shown in Figure 25. In the first donor, the percentage of reactive CD8+ T cells expressing a specific functional phenotype ranged from 0% to around 20%.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Experiment</th>
<th>WT</th>
<th>αD26Y</th>
<th>βL98W</th>
<th>αD26Y/βL98W</th>
<th>αD26Y/αY50A/βL98W</th>
<th>αD26Y/αY50V/βL98W</th>
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Table 14. Summary of Polyfunctional Phenotypes in TCR Transduced CD4+ T cells amongst Six Experiments per each of the DMF5 TCRs.
Figure 25. Impact of DMF5 Mutations in Transduced CD8+ T cells on Polyfunctional Responses against MART-1 (Donor One). The data indicate reactivity against T2 cells loaded with the MART-1 9mer peptide in donor one, experiment one. “Cool plots” were generated to include any functional phenotype present over 0.1%. In the functional phenotypes listed, “+” indicates positive for the marker and “-” indicates negative for the marker. As described in Chapter Two, reactivity is defined after background subtraction, specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). The color depicted in the “cool plot” correlates to the scale on the right. An “X” indicates no T cells were positive (<0.1% after background subtraction) for given functional phenotype. The color white for a given functional phenotype indicates a percentage less than 0.5%. Five prominent phenotypes are highlighted with red boxes.

Among six TCR transduced CD8+ T cell populations, 17 out of the potential 128 polyfunctional phenotypes were detected in at least one DMF5 TCR transduced T cell population, by over 0.1% of TCR transduced CD8+ T cells. Combinations of lysis and Th1, Th2, and Th17 cytokines are all exhibited, except for phenotypes including IL-22. As observed with the CD4+ T cells, differences in the patterns of polyfunctional phenotypes are evident among T cells expressing the different DMF5 TCRs despite seeing the same ligand. For example, the first functional phenotype listed consisted of CD107A+,
IFN-γ⁺, TNF-α⁺, IL-17A⁺ TCR transduced CD8⁺ T cells. This phenotype was exhibited by less than 0.5% of WT TCR, αD26Y TCR, βL98W TCR and αD26Y/αY50A/βL98W TCR expressing T cells, but absent in T cells expressing the αD26Y/βL98W TCR and αD26Y/αY50V/βL98W TCR expressing T cells. Despite the differences observed in polyfunctional patterns amongst the DMF5 TCRs, there were nine functional phenotypes that were reproducibly exhibited by T cells expressing each of the DMF5 TCRs. However, it was notable that a majority of the functional T cells exhibited five prominent functional phenotypes. These five prominent phenotypes (highlighted in red boxes) were comprised of combinations of CD107A, IFN-γ, and TNF-α. Overall, these results indicated in this donor and experiment, TCR transduced T cells exhibited functional phenotypes comprised of lysis and Th₁, Th₂, and Th₁⁷ cytokines. Furthermore, five distinct functional phenotypes were reproducibly exhibited by T cells expressing each of the DMF5 TCRs in response to the MART-1 9mer.

We observed variability and similarity within patterns of polyfunctionality when we compared two independent repeats within the same donor in the TCR transduced CD4⁺ T cells when stimulated with the MART-1 9mer. Therefore, we determined if the patterns described above were reproducible or inconsistent in a second repeat in donor one, with the same TCR transduced CD8⁺ T cell culture, four days later (Figure 49, in the appendix). In the repeat of donor one among six TCR transduced CD8⁺ T cell populations, 21 out of the potential 128 polyfunctional phenotypes were detected in at least one DMF5 TCR transduced T cell population, by over 0.1% of transduced CD8⁺ T cells. Four more phenotypes were exhibited in the second experiment and the percentage of reactive CD8⁺ T cells was reduced from a maximum of 20% to a
maximum of 11%. This indicated the patterns are not completely reproducible in this donor. For example, the IL-4+ only phenotype is exhibited in experiment two and not in experiment one. Furthermore, IL-22 is exhibited in two functional phenotypes, whereas it was absent in experiment one. Despite differences, the five predominant functional phenotypes that were exhibited previously, are reproducibly exhibited in this experiment in T cells expressing each of the DMF5 TCRs. In summary, these two experiments indicated that functional phenotypes are not completely reproducible in the same donor when observed in two independent repeats. Moreover, the five predominant functional phenotypes that were exhibited previously, are reproducibly exhibited by T cells expressing each of the DMF5 TCRs in both experiments.

To determine if donor variability reproducibly occurred in polyclonal PBL-derived CD8+ T cells, we performed this analysis in multiple donors. A representative cool plot for the second donor is shown in Figure 26. In this experiment, the percentage of reactive CD8+ T cells expressing a specific functional phenotype ranged from 0% to around 12%. Among six TCR transduced CD8+ T cell populations, 26 out of the potential 128 polyfunctional phenotypes were detected in at least one DMF5 TCR transduced T cell population, by over 0.1% of TCR transduced CD8+ T cells. Combinations of lysis and Th1, Th2, and Th17 cytokines are all represented, except for phenotypes including IL-22. As observed in the transduced CD4+ T cells, the patterns of polyfunctional phenotypes exhibited was TCR dependent. For example, the first functional phenotype listed consisted of CD107A+, IFN-γ+, TNF-α+, IL-17A+, IL-2+ transduced CD8+ T cells.
Figure 26. Impact of DMF5 Mutations in Transduced CD8+ T cells on Polyfunctional Responses against MART-1 (Donor Two). The data indicate reactivity against T2 cells loaded with the MART-1 9mer peptide in donor two, experiment one. “Cool plots” were generated to include any functional phenotype present over 0.1%. In the functional phenotypes listed, “+” indicates positive for the marker and “-” indicates negative for the marker. As described in Chapter Two, reactivity is defined after background subtraction, specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). The color depicted in the “cool plot” correlates to the scale on the right. An “X” indicates no T cells were positive (<0.1% after background subtraction) for given functional phenotype. The color white for a given functional phenotype indicates a percentage less than 0.5%. Five prominent phenotypes are highlighted with red boxes.

This phenotype was exhibited by less than 0.5% of αD26Y/αY50A/βL98W TCR and αD26Y/αY50V/βL98W TCR expressing T cells, and not exhibited by T cells expressing each of the other DMF5 TCRs. The five prominent phenotypes (highlighted in red boxes) comprised of combinations of CD107A, IFN-γ, and TNF-α were also observed in this donor and experiment. Overall, these results indicated transduced T cells were capable of exhibiting functional phenotypes comprised of lysis and Th1, Th2, and Th17 cytokines. Moreover, five functional phenotypes were reproducibly exhibited by T cells expressing each of the DMF5 TCRs in response to the MART-1 9mer.
When previously comparing two independent repeats in the same donors, we observed variability and similarity within patterns of polyfunctionality elicited by TCR transduced CD4+ T cells when stimulated with the MART-1 9mer. Therefore, we determined if the patterns described above were reproducible in a second repeat in donor two, with the same TCR transduced CD8+ T cell culture, four days later (Figure 50, in the appendix). In the repeat of donor two among six TCR transduced CD8+ T cell populations, 48 out of the potential 128 polyfunctional phenotypes were detected in at least one DMF5 TCR transduced T cell population, by over 0.1% of transduced CD8+ T cells. 21 more phenotypes were represented in the second experiment. This indicated the patterns are not completely reproducible in this donor. Here, the five predominant functional phenotypes that were exhibited in the first experiment are reproducibly exhibited. However, in addition to these five, there are additional polyfunctional phenotypes exhibited by the transduced T cells expressing each of the DMF5 TCRs. Furthermore, IL-22 is exhibited in functional phenotypes, whereas it was not in experiment one. In summary, these two repeats indicated that functional phenotypes are not completely reproducible in the same donor when observed in two independent repeats. However, the five predominant functional phenotypes that were exhibited in the first experiment are reproducibly exhibited by T cells expressing each of the DMF5 TCRs.

To determine if donor variability was again observed with another donor in TCR transduced CD8+ T cells when stimulated with the MART-1 9mer, we performed this analysis in a third donor. A representative cool plot for the third donor is shown in Figure 27.
Figure 27. Impact of DMF5 Mutations in Transduced CD8<sup>+</sup> T cells on Polyfunctional Responses against MART-1 (Donor Three). The data indicate reactivity against T2 cells loaded with the MART-1 9mer peptide in donor three, experiment one. “Cool plots” were generated to include any functional phenotype present over 0.1%. In the functional phenotypes listed, “+” indicates positive for the marker and “−” indicates negative for the marker. As described in Chapter Two, reactivity is defined after background subtraction, specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). The color depicted in the “cool plot” correlates to the scale on the right. An “X” indicates no T cells were positive (<0.1% after background subtraction, as described in Chapter Two) for given functional phenotype. The color white for a given functional phenotype indicates a percentage less than 0.5%. Five prominent phenotypes are highlighted with red boxes.

In donor three, the percentage of reactive CD8<sup>+</sup> T cells expressing a specific functional phenotype ranged from 0% to around 13%. Among six TCR transduced CD8<sup>+</sup> T cell populations, 30 out of the potential 128 polyfunctional phenotypes were detected in at least one DMF5 TCR transduced T cell population, by over 0.1% of TCR transduced CD8<sup>+</sup> T cells. Differences in polyfunctional phenotypes are evident among T cells expressing the different DMF5 TCRs despite seeing the same ligand. For example, the first phenotype consisted of CD107A<sup>+</sup>, IFN-γ<sup>+</sup>, TNF-α<sup>+</sup>, IL-22<sup>+</sup> CD8<sup>+</sup> transduced T cells. This phenotype was exhibited by less than 0.5% of βL98W TCR expressing T cells, but
absent in T cells expressing each of the other DMF5 TCRs. Despite the differences among T cells expressing each of the DMF5 TCRs, the five prominent phenotypes (highlighted in red boxes) comprised of combinations of CD107A, IFN-γ, and TNF-α were also observed in this donor and experiment (except in T cells expressing the αD26Y/βL98W TCR). Overall, this experiment demonstrated the polyfunctionality of transduced CD8+ T cells and the variability of the functional phenotypes demonstrated in T cells expressing different DMF5 TCRs despite presentation of the same ligand. Furthermore, the five predominant functional phenotypes that were exhibited in the first donor are reproducibly exhibited.

We observed variability and similarity within patterns of polyfunctionality elicited by TCR transduced T cells when stimulated with the MART-1 9mer when we compared two independent repeats in the first two donors. Therefore, we determined if the patterns described above were reproducible in a second repeat in the third donor, with the same TCR transduced T cell culture, four days later (Figure 51, in the appendix). In the repeat of donor three among six TCR transduced T cell populations, 42 out of the potential 128 polyfunctional phenotypes were detected in over 0.1% of TCR transduced CD8+ T cells in this donor and this experiment. 21 more phenotypes were represented in the second experiment and the maximum percentage of reactive CD8+ T cells was increased from a maximum of 13% to about 16%. This indicated the patterns are not completely reproducible in this donor. For example, the IFN-γ+, TNF-α+, IL-2+ phenotype is exhibited by T cells expressing each DMF5 TCR in the second experiment, but is only exhibited by T cells expressing the WT TCR, βL98W TCR, and αD26Y/αY50V/βL98W TCR in experiment one. Despite differences in the patterns amongst the different DMF5
TCRs, the five predominant functional phenotypes that were exhibited previously are reproducibly exhibited in this second experiment. In summary, these two repeats indicated that functional phenotypes are not completely reproducible in the same donor when observed in two independent repeats. Moreover, the five predominant functional phenotypes that were exhibited previously, are reproducibly exhibited in T cells expressing each of the DMF5 TCRs.

Thus far, we demonstrated that transduced CD8+ T cells were polyfunctional against the MART-1 9mer. There are a few overarching conclusions that were made based upon the polyfunctional responses of T cells expressing each of the DMF5 TCRs against the MART-1 9mer in different experiments and donors. The first set of conclusions encompassed variability and similarity amongst polyfunctional patterns between donors and experiments. Table 15 depicts a numerical summary of the number of functional phenotypes observed and their reproducibility amongst each experiment and donor. First, as with the CD4+ T cells, the number of polyfunctional phenotypes exhibited was donor and experiment dependent. Secondly, as with the CD4+ T cells, the number of reproducible polyfunctional phenotypes amongst T cells expressing each of the DMF5 T cells were experiment dependent. Thirdly, the number of reproducible functional phenotypes exhibited in T cells expressing each of the DMF5 TCRs was donor dependent. For example, the number of unique reproducible functional phenotypes in T cells expressing each of the DMF5 TCRs was different in every donor (donor 1 – five, donor 2 – eight, donor 3 – five). Fourthly, five functional phenotypes were exhibited by CD8+ T cells expressing each of the DMF5 TCRs in every donor and in every experiment (except with one TCR in donor three, experiment one).
Table 15. Summary of Polyfunctional Phenotypes in TCR Transduced CD8+ T cells amongst Six Experiments. *Reproducible phenotypes with every DMF5 TCR indicate that within a specific experiment, all six TCR transduced CD8+ T cell populations (WT DMF5 TCR and each mutant DMF5 TCR) exhibited the functional phenotype.
**Reproducible phenotypes with every DMF5 TCR within donor indicate that within a specific donor, all six TCR transduced CD8+ T cell populations (WT DMF5 TCR and each mutant DMF5 TCR) exhibited the functional phenotype in both experimental repeats.

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<th>Experiment</th>
<th>Total # of phenotypes exhibited</th>
<th># of phenotypes exhibited with every DMF5 TCR*</th>
<th># of reproducible phenotypes with every DMF5 TCR within donor**</th>
<th>reproducible phenotypes within CD8+ T cells</th>
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| 1     | 1          | 17                             | 9                                             | 5                                                             | 1. CD107A+, IFN-γ+, TNF-α+  
2. IFN-γ+, TNF-α+  
3. CD107A+, IFN-γ+  
4. IFN-γ+  
5. CD107A+ (except with one TCR in donor 3, experiment 1) |
|       | 2          | 21                             | 8                                             |                                                               |
| 2     | 1          | 26                             | 10                                            | 8                                                             |                                                          |
|       | 2          | 48                             | 15                                            |                                                               |
| 3     | 1          | 30                             | 7                                             | 5                                                             |                                                          |
|       | 2          | 42                             | 10                                            |                                                               |

The five phenotypes identified are: 1.) CD107A+, IFN-γ+, TNF-α+, 2.) IFN-γ+, TNF-α+, 3.) CD107A+, IFN-γ+, 4.) IFN-γ+ only, and 5.) CD107A+ only. This indicated that in CD8+ T cells expressing each of the DMF5 TCRs will be lytic, produce type 1 cytokines, or do both in response to the MART-1 9mer. In summary, these data indicated polyfunctional responses of CD8+ T cells are dependent upon the TCR, experiment, and donor.

However, we demonstrated that in response to the MART-1 9mer, T cells expressing each of the DMF5 TCRs almost always exhibited a lytic and/or IFN-γ+ and TNF-α+ phenotype.

The second set of conclusions encompassed variability and similarity amongst polyfunctional patterns between donors and experiments in regards to each of the DMF5 TCRs. Table 16 depicts a numerical summary of the number of functional phenotypes observed amongst T cells expressing each of the DMF5 TCRs in each
First, the number of polyfunctional phenotypes exhibited is DMF5 TCR dependent. Secondly, the number of polyfunctional phenotypes exhibited amongst T cells expressing any given DMF5 TCR is experiment and donor dependent. For example, T cells expressing the WT DMF5 TCR exhibited a different number of polyfunctional phenotypes in each of the six experiments. Thirdly, like the CD4+ T cells, the total number and the average number of functional phenotypes amongst the six experiments, generally trends towards a correlation between number of polyfunctional phenotypes and the overall percentages of reactive TCR transduced CD8+ T cells. For example, in Figure 20, CD8+ T cells expressing each of the DMF5 TCRs exhibited a trend towards a reduction (statistically significant in T cells expressing the αD26Y/βL98W TCR or αD26Y/αY50A/βL98W TCR) in the percentage of MART-1 9mer reactive T cells compared to T cells expressing the WT TCR. With the one exception of T cells expressing the αD26Y/αY50V/βL98W TCR, the total number and the average number of polyfunctional phenotypes exhibited by T cells expressing each of the DMF5 TCRs trends towards a reduction compared to T cells expressing the WT TCR amongst the six experiments. Conclusively, these results indicated polyfunctional phenotypes exhibited amongst T cells expressing each of the DMF5 TCRs is TCR, experiment, and donor dependent. However, CD8+ T cells expressing each of the DMF5 TCRs nearly always exhibited the same five functional phenotypes in response to the MART-1 9mer. Lastly, the number of different functional phenotypes exhibited generally correlated to the overall patterns observed with the percentages of MART-1 9mer reactive TCR transduced CD8+ T cells.
### Table 16. Summary of Polyfunctional Phenotypes in TCR Transduced CD8⁺ T cells amongst Six Experiments per each of the DMF5 TCRs.

We have independently concluded that the polyfunctional responses of CD4⁺ and CD8⁺ T cells expressing either the WT DMF5 or each of the mutant DMF5 TCRs against the MART-1 9mer. Many of the conclusions remained consistent between CD4⁺ and CD8⁺ T cells. However, there are also some notable differences. For example, the magnitudes of the percentages of T cells expressing a given phenotype in CD8⁺ T cells was increased compared to the CD4⁺ T cells. This was expected due to the contribution of CD8 in stabilization and in signaling. Secondly, with the one exception of T cells expressing the αD26Y/αY50A/βL98W TCR, on average, TCR transduced CD4⁺ T cells exhibited more polyfunctional phenotypes than TCR transduced CD8⁺ T cells. This indicated that the response of CD8⁺ T cells are less heterogeneous than CD4⁺ T cells. Additionally, different polyfunctional phenotypes were reproducibly exhibited in T cells expressing each of the DMF5 TCRs between the CD4⁺ and CD8⁺ T cells. Notably, CD107A⁺ only phenotypes were more prevalent in the CD8⁺ T cells than the CD4⁺ T cells. This is consistent with the knowledge in the field that CD8⁺ T cells are generally

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more lytic than CD4+ T cells [436]. Overall, CD4+ and CD8+ T cells were polyfunctional and the polyfunctional phenotypes displayed were dependent upon the donor, experiment, and DMF5 TCR. Lastly, the number of polyfunctional phenotypes exhibited against the MART-1 9mer with a given DMF5 TCR, generally correlated to the pattern associated with the percentages of MART-1 9mer reactive T cells.

Thus far we have focused on polyfunctional responses against the MART-1 9mer peptide. Furthermore, we observed five prominent and reproducible functional patterns in CD8+ T cells expressing each of the DMF5 TCRs. However, altered-peptide ligands have been shown to elicit different polyfunctional phenotypes in CD8+ T cells [95]. Therefore, we wanted to determine if these five prominent functional patterns were observed amongst T cells expressing each of the different DMF5 TCRs. Polyfunctional responses would be important in CD4+ T cells, however, since only T cells expressing the αD26Y TCR and αD26Y/βL98W TCR elicited significant reactivity against the MART-1 homologs in CD4+ T cells (based on Figure 19), we focused our attention on the CD8+ T cells. Therefore, we sought to determine how the DMF5 TCRs altered polyfunctional phenotypes against the MART-1 homologs in DMF5 TCR transduced CD8+ T cells. These five functional phenotypes were also apparent in polyfunctional responses against the MART-1 homologs amongst T cells expressing the different DMF5 TCRs in different donors and experiments. For proof of concept, representative cool plots from the three donors depicting polyfunctional responses against M. *tuberculosis* I protein, HSV-1 glycoprotein III, and ADP-ribose diphosphatase are shown in Figures 28-30. These three peptides were chosen as examples for proof of concept
because they were the top three most recognized MART-1 homologs (based on Figure 20).

Reproducible conclusions are evident when observing the polyfunctional responses against the M. *tuberculosis* protein I (Figure 28). Specifically, the TCR transduced CD8\(^+\) T cells are polyfunctional, and polyfunctional responses are DMF5 TCR and donor dependent. However, the five prominent phenotypes previously described in CD8\(^+\) T cell responses against the MART-1 9mer, are evident here, with the different ligand. These results indicated a reproducible pattern of polyfunctional responses with different ligands. Furthermore, CD8\(^+\) T cells expressing the WT TCR exhibited 11, 11, and 12 functional phenotypes against the M. *tuberculosis* protein I, per each donor, respectively. CD8\(^+\) T cells expressing the αD26Y TCR exhibited more functional phenotypes compared to T cells expressing the WT TCR at 13, 19, and 18 functional phenotypes per each donor, respectively. CD8\(^+\) T cells expressing the αD26Y/αY50A/βL98W TCR exhibited fewer functional phenotypes than T cells expressing the WT TCR at 3, 7, and 2 functional phenotypes per each donor, respectively. These results coincided with the results in Figure 20 depicting a statistically significant increase and decrease in the percentages of M. *tuberculosis* protein I reactive CD8\(^+\) T cells in T cells expressing the αD26Y TCR and αD26Y/αY50A/βL98W TCR, respectively, compared to T cells expressing the WT TCR. Specifically, these results indicated that a greater percentage of TCR transduced M. *tuberculosis* protein I reactive CD8\(^+\) T cells correlates to a greater number of functional phenotypes exhibited.
Figure 28. Impact of DMF5 Mutations in TCR Transduced CD8+ T cells on Polyfunctional Responses against M. tuberculosis protein I. “Cool plots” were generated to include any functional phenotype present over 0.1%. In the functional phenotypes listed, “+” indicates positive for the marker and “-“ indicates negative for the marker. As described in Chapter Two, reactivity is defined after background subtraction, specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). The color depicted in the “cool plot” correlates to the scale on the right. An “X” indicates no T cells were positive (≤0.1% after background subtraction) for given functional phenotype. The color white for a given functional phenotype indicates a percentage less than 0.2% (a) or 0.5% (b and c). Five prominent phenotypes are highlighted with red boxes. (a) donor one (b) donor two (c) donor three.

Overall, T cell polyfunctionality and the five predominant polyfunctional phenotypes were observed with CD8+ T cells expressing each of the different DMF5 TCRs against a different ligand.

We next observed the polyfunctional patterns demonstrated in response to the HSV-1 glycoprotein III. Polyfunctional responses against the HSV-1 glycoprotein III indicated reproducible observations that have been previously described (Figure 29). Moreover, the TCR transduced CD8+ T cells are polyfunctional, and polyfunctional responses are DMF5 TCR and donor dependent. Nonetheless, the five prominent phenotypes previously described in CD8+ T cell responses against the MART-1 9mer, are evident here, with the different ligand. Furthermore, CD8+ T cells expressing the WT TCR exhibited 7, 10, and 13 functional phenotypes against the HSV-1 glycoprotein III, per each donor, respectively. CD8+ T cells expressing the αD26Y TCR exhibited more functional phenotypes compared to T cells expressing the WT TCR at 10, 20, and 36 functional phenotypes per each donor, respectively.
Figure 29. Impact of DMF5 Mutations in TCR Transduced CD8+ T cells on Polyfunctional Responses against HSV-1 glycoprotein III. “Cool plots” were generated to include any functional phenotype present over 0.1%. In the functional phenotypes listed, “+” indicates positive for the marker and “-“ indicates negative for the marker. As described in Chapter Two, reactivity is defined after background subtraction, specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). The color depicted in the “cool plot” correlates to the scale on the right. An “X” indicates no T cells were positive (<0.1% after background subtraction) for given functional phenotype. The color white for a given functional phenotype indicates a percentage less than (a) 0.2% or (b and c) 0.5%. Five prominent phenotypes are highlighted with red boxes. (a) donor one (b) donor two (c) donor three.

CD8+ T cells expressing the αD26Y/αY50A/βL98W TCR exhibited fewer functional phenotypes than T cells expressing the WT TCR at 2, 4, and 6 functional phenotypes per each donor, respectively. These results coincided with the results in Figure 20 depicting a statistically significant increase and decrease in the percentages of HSV-1 glycoprotein III reactive CD8+ T cells in T cells expressing the αD26Y TCR and αD26Y/αY50A/βL98W TCR, respectively, compared to T cells expressing the WT TCR. Specifically, these results indicated that a greater percentage of TCR transduced HSV-glycoprotein III reactive CD8+ T cells correlates to a greater number of functional phenotypes exhibited. Overall, T cell polyfunctionality and the five predominant polyfunctional phenotypes were observed with CD8+ T cells expressing each of the different TCRs against a different ligand.

Lastly, we demonstrated that polyfunctional responses against the ADP-ribose diphosphatase are dependent upon the DMF5 TCR and the donor (Figure 30). However, the five prominent phenotypes previously described in CD8+ T cell responses are evident here, with the different MART-1 homolog.
**Figure 30. Impact of DMF5 Mutations in TCR Transduced CD8+ T cells on Polyfunctional Responses against ADP-ribose diphosphatase.** "Cool plots" were generated to include any functional phenotype present over 0.1%. In the functional phenotypes listed, “+” indicates positive for the marker and “-” indicates negative for the marker. As described in Chapter Two, reactivity is defined after background subtraction, specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). The color depicted in the “cool plot” correlates to the scale on the right. An “X” indicates no T cells were positive (≤0.1% after background subtraction) for given functional phenotype. The color white for a given functional phenotype indicates a percentage less than 0.2%. Five prominent phenotypes are highlighted with red boxes. (a) donor one (b) donor two (c) donor three.

Furthermore, CD8+ T cells expressing the WT TCR exhibited 7, 14, and 11 functional phenotypes against the ADP-ribose diphosphatase, per each donor, respectively. CD8+ T cells expressing the αD26Y TCR exhibited an equal number or more functional phenotypes compared to T cells expressing the WT TCR at 7, 16, and 27 functional phenotypes per each donor, respectively. CD8+ T cells expressing the αD26Y/αY50A/βL98W TCR exhibited fewer functional phenotypes than T cells expressing the WT TCR at 4, 11, and 4 functional phenotypes per each donor, respectively. These results coincided with the results in Figure 20 depicting a trend towards an increase and decrease in the percentages of ADP-ribose diphosphatase reactive CD8+ T cells in T cells expressing the αD26Y TCR and αD26Y/αY50A/βL98W TCR, respectively, compared to T cells expressing the WT TCR. As observed with the MART-1 9mer, M. tuberculosis protein I, and HSV-1 glycoprotein III, these results indicated that a greater percentage of TCR transduced ADP-ribose diphosphatase reactive CD8+ T cells correlates to a greater number of functional phenotypes exhibited. Overall, T cell polyfunctionality and the five predominant polyfunctional phenotypes
were observed with CD8+ T cells expressing each of the different TCRs against a
different ligand.

In light of all the polyfunctional results against different ligands from CD8+ T cells
expressing each of the DMF5 TCRs in different experiments and donors, there are
reproducible and overarching conclusions that can be made. First, it is evident that
polyfunctional responses are DMF5 TCR, experiment, donor, and ligand dependent.
However, amongst the variability, five reproducible functional phenotypes are frequently
exhibited by T cells expressing each of DMF5 TCRs against different ligands, in
different donors and experiments. Secondly, the number of polyfunctional phenotypes
exhibited against a given ligand, generally correlated to the patterns observed in the
percentages of ligand reactive T cells. This indicated the more antigen reactive T cells
there are, the more polyfunctional phenotypes they will exhibit. Overall, these data
suggested that patterns in polyfunctionality are evident, but specific functional
phenotypes are ultimately dependent upon the TCR, ligand, experiment, and donor.

Impact of Mutant DMF5 TCRs on Cross-Reactivity within Major Polyfunctional
Phenotypes

Thus far, we have described patterns of polyfunctionality in CD8+ T cells against
four targets in the form of cool plots. Analysis via cool plots is advantageous to visually
examine all the polyfunctional phenotypes exhibited and to visually examine how
mutations in the DMF5 TCR altered polyfunctional phenotypes against a specific ligand.
However, cool plots did not feasibly allow us to quantitatively determine how mutations
in the DMF5 TCR altered cross-reactivity patterns against all the MART-1 homologs
within specific functional phenotypes. Nonetheless, this analysis is of importance, since
our previous conclusions on cross-reactivity were centered on antigen reactivity based upon expression of any one or more functional marker. Through the analysis of cool plots, we have identified five functional phenotypes that were predominantly expressed among CD8+ T cells expressing each of the DMF5 TCRs, against different ligands, and in different experiments and donors. The five phenotypes identified were: 1.) CD107A+, IFN-γ+, TNF-α+, 2.) IFN-γ+, TNF-α+, 3.) CD107A+, IFN-γ+, 4.) IFN-γ+ only, and 5.) CD107A+ only. Seven potential combinations of functional phenotypes were possible with these three functional markers. CD107A+, TNF-α+ and TNF-α+ only are the two phenotypes missing from these combinations. However, these two phenotypes were not reproducibly exhibited in our analysis. This indicated that generally, if TNF-α was produced, IFN-γ was also produced. Based upon these reproducible findings, we next determined if the cross-reactive patterns observed against the MART-1 homologs in Figure 20 were reproducible when focusing on each of these five predominant functional phenotypes. We first examined TCR transduced CD8+ T cells expressing CD107A, IFN-γ, and TNF-α (Figure 31). The average of six experiments found 9.2% of T cells expressing the WT TCR exhibited the CD107A+, IFN-γ+, TNF-α+ polyfunctional phenotype in response to targets loaded with the MART-1 9mer. Furthermore, T cells expressing the WT TCR recognized four of the MART-1 homologs, ranging between 1% and 2.6% reactive T cells. We observed similar patterns in reactivity against MART-1 and the MART-1 homologs with T cells expressing the mutant DMF5 TCRs that were previously observed when examining reactivity based on any one or more functional marker.
Figure 31. Impact of DMF5 Mutations on Cross-Reactivity in TCR Transduced CD8+ T cells Expressing the CD107A+, IFN-γ+, TNF-α+ Phenotype. Percent reactive CD8+ T cells expressing the CD107A+, IFN-γ+, TNF-α+ functional phenotype were determined using data generated from the six cool plots. Self-peptides are indicated by a black triangle. Data represent the average of 6 experiments (three donors, two independent repeats) and error bars represent the standard error of the mean. As described in Chapter Two, reactivity is defined after background subtraction, specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). **** P <0.0001; ***P < 0.001; **P < 0.01; *P < 0.05 when the percentage of antigen reactive T cells against each peptide with a given TCR was compared to the percentage of antigen reactive T cells to the respective peptide with the WT TCR by two-way ANOVA using Sidak’s multiple comparisons tests.

Specifically, the percentage of MART-1 9mer reactive T cells was comparable between T cells expressing the αD26Y TCR and T cells expressing the WT TCR (9.6% vs. 9.2%). Furthermore, the percentages of αD26Y TCR expressing MART-1 homolog reactive T cells was increased compared to T cells expressing the WT TCR. Statistical significance was reached in HSV-1 glycoprotein III (10%), M. tuberculosis protein I (8.3%), human CD9 (3.4%) and GPCR 3 (4.5%). In T cells expressing the βL98W TCR,
the percentage of MART-1 9mer (9.1% vs. 9.2%) and MART-1 homolog (0.1% - 2.6% vs. 1% - 2.6%) reactive T cells was comparable to T cells expressing the WT TCR. However, there was one apparent exception, although not significant, the percentage of antigen reactive βL98W TCR expressing T cells was increased against human CD9, compared to T cells expressing the WT TCR (1.4% vs. 0%). This coincided with our previous conclusions made in Figure 20. T cells expressing the αD26Y/βL98W TCR demonstrated a statistically significant reduction in the percentage of MART-1 9mer reactive T cells compared to T cells expressing the WT TCR (2% vs. 9.2%), consistent with previous findings. There were no statistically significant changes in the percentage of MART-1 homolog reactive T cells, consistent with previous findings. Although not statistically significant compared to T cells expressing the WT TCR, T cells expressing the αD26Y/αY50A/βL98W TCR exhibited a trend towards a reduction in the percentage of MART-1 9mer reactive T cells (9.2% vs. 6.5%) and MART-1 homolog reactive T cells (1% to 2.6% vs. 0.28% to 1.1%). Lastly, there were no statistically significant differences between the WT TCR and the αD26Y/αY50V/βL98W TCR in terms of the percentages of MART-1 9mer (9.2% vs. 7.7%) and MART-1 homolog reactive (1% to 2.6% vs. 0.1% to 2.5%) TCR transduced CD8+ T cells, consistent with previous findings in Figure 20. Overall, when examining the percentages of MART-1 and MART-1 homolog reactive T cells exhibiting the CD107A+, IFN-γ+, TNF-α+ polyfunctional phenotype, we generated conclusions that were similar to the conclusions we generated from Figure 20. Mainly, the percentages of MART-1 homolog reactive TCR transduced CD8+ T cells were generally enhanced with the αD26Y TCR and reduced with the αD26Y/αY50A/βL98W TCR, compared to the WT DMF5 TCR. Furthermore, T cells expressing the
αD26Y/αY50A/βL98W TCR exhibited a trend towards a reduction in the percentage of MART-1 9mer and MART-1 homolog reactive T cells compared to T cells expressing the WT TCR. These results indicated that patterns in MART-1 9mer and MART-1 homolog reactivity were comparable when observing the overall percentages of antigen reactive TCR transduced CD8\(^+\) T cells (Figure 20), or the percentages of antigen reactive TCR transduced CD8\(^+\) T cells expressing a specific polyfunctional phenotype (Figure 31). We next determined if the patterns observed in MART-1 9mer and MART-1 homolog reactivity were reproducible in the other four functional phenotypes: IFN-γ\(^+\), TNF-α\(^+\) (Figure 32), CD107A\(^+\), IFN-γ\(^+\) (Figure 33), IFN-γ\(^+\) only (Figure 34), and CD107A\(^+\) only (Figure 35).

Our observations from these data indicated that patterns in antigen recognition among different DMF5 TCR transduced CD8\(^+\) T cell populations are frequently reproducible when examining antigen reactivity in terms of the total percentage of antigen reactive T cells (Figure 20), or when examining antigen reactivity in terms of expressing individual functional phenotypes (Figures 31-35). Moreover, CD8\(^+\) T cells expressing the WT TCR are cross-reactive, as they recognized between four and nine MART-1 homologs within each functional phenotype (Figures 31-35). Overall, CD8\(^+\) T cells expressing each of the mutant DMF5 TCRs generally exhibited similar patterns in the percentages of MART-1 9mer and MART-1 homolog reactive T cells among the five specific functional phenotypes, compared to T cells expressing the WT TCR.
Figure 3. Impact of DMF5 Mutations on Cross-Reactivity in TCR Transduced CD8+ T cells Expressing the IFN-γ+, TNF-α+ Phenotype. Percent reactive CD8+ T cells expressing the IFN-γ+, TNF-α+ functional phenotype were determined using data generated from the six cool plots. Self-peptides are indicated by a black triangle. Data represent the average of 6 experiments (three donors, two independent repeats) and error bars represent the standard error of the mean. As described in Chapter Two, reactivity is defined after background subtraction, specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). **** P <0.0001; ***P < 0.001; **P < 0.01; *P < 0.05 when the percentage of antigen reactive T cells against each peptide with a given TCR was compared to the percentage of antigen reactive T cells to the respective peptide with the WT TCR by two-way ANOVA using Sidak’s multiple comparisons tests.
Figure 33. Impact of DMF5 Mutations on Cross-Reactivity in TCR Transduced CD8+ T cells Expressing the CD107A+, IFN-γ+ Phenotype. Percent reactive CD8+ T cells expressing the CD107A+, IFN-γ+ functional phenotype were determined using data generated from the six cool plots. Self-peptides are indicated by a black triangle. Data represent the average of 6 experiments (three donors, two independent repeats) and error bars represent the standard error of the mean. As described in Chapter Two, reactivity is defined after background subtraction, specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). **** P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05 when the percentage of antigen reactive T cells against each peptide with a given TCR was compared to the percentage of antigen reactive T cells to the respective peptide with the WT TCR by two-way ANOVA using Sidak’s multiple comparisons tests.
Figure 34. Impact of DMF5 Mutations on Cross-Reactivity in TCR Transduced CD8+ T cells Expressing the IFN-γ only Phenotype. Percent reactive CD8+ T cells expressing the IFN-γ only functional phenotype were determined using data generated from the six cool plots. Self-peptides are indicated by a black triangle. Data represent the average of 6 experiments (three donors, two independent repeats) and error bars represent the standard error of the mean. As described in Chapter Two, reactivity is defined after background subtraction, specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). **** P <0.0001; ***P < 0.001; **P < 0.01; *P < 0.05 when the percentage of antigen reactive T cells against each peptide with a given TCR was compared to the percentage of antigen reactive T cells to the respective peptide with the WT TCR by two-way ANOVA using Sidak’s multiple comparisons tests.
Figure 35. Impact of DMF5 Mutations on Cross-Reactivity in TCR Transduced CD8\(^+\) T cells Expressing the CD107A\(^+\) only Phenotype. Percent reactive CD8\(^+\) T cells expressing the CD107A\(^+\) only functional phenotype were determined using data generated from the six cool plots. Self-peptides are indicated by a black triangle. Data represent the average of 6 experiments (three donors, two independent repeats) and error bars represent the standard error of the mean. As described in Chapter Two, reactivity is defined after background subtraction, specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide).

Specifically, T cells expressing the \(\alpha D26Y\) TCR exhibited more MART-1 homolog reactive T cells compared to T cells expressing the WT TCR, with statistical significance reached with some MART-1 homologs in four of the five functional phenotypes (Figures 31 and 33-35). It was notable that T cells expressing the \(\alpha D26Y\) TCR exhibited significantly more MART-1 9mer reactive T cells in the CD107A\(^+\) only (Figure 35) and CD107A\(^+\), IFN-\(\gamma^+\) (Figure 33) phenotype. This was not observed in Figure 20 when examining the total percentage of MART-1 9mer reactive CD8\(^+\) T cells. This indicated
that these patterns of MART-1 9mer reactivity are not 100% reproducible within individual phenotypes. Overall, these results indicated that the tyrosine mutation at position 26 in the TCR alpha chain did not enhance MART-1 9mer antigen specificity in TCR transduced CD8+ T cells expressing specific functional phenotypes. However, compared to the WT TCR, a statistically significant increase in the percentages of MART-1 9mer reactive CD8+ T cells was observed in αD26Y TCR transduced CD8+ T cells expressing the CD107A+ only and CD107A+, IFN-γ+ phenotypes.

We next examined reproducible patterns in MART-1 9mer and MART-1 homolog reactivity with CD8+ T cells expressing the βL98W TCR. Specifically, the percentages of MART-1 9mer reactive T cells were comparable between βL98W TCR and WT TCR expressing CD8+ T cells amongst the five functional phenotypes (Figures 31-35). Additionally, the percentages of MART-1 homolog reactive T cells were comparable between βL98W TCR and WT TCR expressing CD8+ T cells amongst the five functional phenotypes (Figures 31-35). Although not significant, one notable difference was a trend towards an increase in human CD9 reactive T cells expressing the βL98W TCR compared to T cells expressing the WT TCR amongst the five functional phenotypes (Figures 31-35). This was also previously demonstrated in Figure 20. Overall, these results indicated that the tryptophan mutation at position 98 in the TCR beta chain did not enhance MART-1 9mer antigen specificity in TCR transduced CD8+ T cells expressing specific functional phenotypes.

We next elucidated reproducible patterns in MART-1 9mer and MART-1 homolog reactivity with T cells expressing the αD26Y/βL98W TCR. Specifically, the percentages of MART-1 9mer reactive T cells were statistically significantly reduced in T cells
expressing the αD26Y/βL98W TCR compared to T cells expressing the WT TCR in four of the five functional phenotypes (Figures 31-34). Furthermore, the percentages of MART-1 homolog reactive T cells were comparable, with no statistically significant differences (Figures 31-35). Overall, the combination of the αD26Y and βL98W TCR mutations did not enhance MART-1 9mer antigen specificity and attenuated MART-1 reactivity in TCR transduced CD8+ T cells expressing specific functional phenotypes.

We next examined comparable patterns in MART-1 9mer and MART-1 homolog reactivity with T cells expressing the αD26Y/αY50A/βL98W TCR. Specifically, T cells expressing the αD26Y/αY50A/βL98W TCR significantly reduced the percentages of MART-1 reactive CD8+ T cells in two of the five functional phenotypes, compared to the WT TCR (Figures 33-34). Furthermore, T cells expressing the αD26Y/αY50A/βL98W TCR exhibited a trend towards a reduction in the percentages of MART-1 homolog reactive T cells compared to T cells expressing the WT TCR (Figures 31-32, and 35), reaching statistical significance with some of the MART-1 homologs in two of the five functional phenotypes (Figures 33-34). In summary, the addition of the alanine TCR mutation at position 50 in the TCR alpha chain to the αD26Y/βL98W TCR resulted in an overall trend in reduced MART-1 9mer and MART-1 homolog reactivity in TCR transduced CD8+ T cells expressing specific functional phenotypes.

Lastly, there were no significant differences in the percentages of MART-1 9mer and MART-1 homolog reactive αD26Y/αY50V/βL98W TCR or WT TCR expressing T cells amongst the five functional phenotypes (Figures 31-35). Overall, the valine TCR mutation at position 50 in the TCR alpha did not have as much as an impact on MART-1
9mer and MART-1 homolog reactivity as the alanine TCR mutation in TCR transduced CD8+ T cells expressing specific functional phenotypes.

Based on all the data observing antigen specificity and cross-reactivity in DMF5 TCR transduced CD8+ T cells expressing specific functional phenotypes, a few comprehensive conclusions were generated. First, trends in MART-1 9mer and MART-1 homolog recognition amongst T cells expressing each of the DMF5 TCRs were commonly reproducible whether examining the total percentages of antigen reactive CD8+ T cells (Figure 20), or the percentages of antigen reactive CD8+ T cells expressing a specific functional phenotype (Figures 31-35). However, these trends were not 100% reproducible with each DMF5 TCR in each individual phenotype. This suggested that observing antigen reactivity in regards to exhibiting any functional phenotype (Figure 20) gives a baseline for the total percentage of antigen reactive T cells. Subsequently, it is plausible to observe minor variation around that baseline within individual functional phenotypes (Figures 31-35). In summary, these results indicated the patterns of MART-1 and MART-1 homolog reactivity elicited with T cells expressing each of the different DMF5 TCRs are generally or broadly reproducible when comparing the total percentage of antigen reactive T cells or the percentage of antigen reactive T cells within an individual phenotype.

It was of interest to determine if the patterns of the CD107A+ only phenotype (Figure 35) in response to the MART-1 9mer correlated to patterns in lysis of MEL 624 in Figure 17. Our results indicated that the percentages of CD107A+ only CD8+ T cells in response to the MART-1 9mer generally correlated with cytotoxicity. For example, compared to T cells expressing the WT TCR, αD26Y TCR expressing T cells exhibited
a statistically significant increase in MEL 624 killing (Figure 17) and exhibited a statistically significant increase in the percentage of CD107A\(^+\) only CD8\(^+\) T cells in response to the MART-1 9mer (Figure 35). Furthermore, compared to T cells expressing WT TCR, T cells expressing the βL98W TCR, αD26Y/αY50A/βL98W TCR, or αD26Y/αY50V/βL98W TCR exhibited comparable, or a trend towards an increase in MEL 624 killing (Figure 17) and exhibited similar percentages of CD107A\(^+\) only CD8\(^+\) T cells in response to the MART-1 9mer (Figure 35). However, there was one exception to this correlation. αD26Y/βL98W TCR expressing T cells exhibited a statistically significant increase in MEL 624 killing (Figure 17), but exhibited a trend towards a decrease in the percentages of CD107A\(^+\) only CD8\(^+\) T cells compared to CD8\(^+\) T cells expressing the WT TCR (Figure 35). One caveat is that the percentage of CD107A\(^+\) only CD8\(^+\) T cells was much lower than the percentages of functional phenotypes exhibiting both CD107A and cytokines. This indicated CD107A\(^+\) T cells, or T cells that have potentially killed, generally also expressed cytokines. Therefore, we examined the total percentages of CD107A\(^+\) T cells against the MART-1 9mer to determine if there was a correlation with MEL 624 lysis (Figure 36). In CD8\(^+\) T cells, T cells expressing the αD26Y/βL98W TCR exhibited a statistically significantly lower percentage of MART-1 9mer reactive CD107A\(^+\) T cells compared to T cells expressing the WT TCR (Figure 36A). This did not correlate with lysis of MEL 624 in Figure 17. Although not statistically significant compared to CD8\(^+\) T cells expressing the WT TCR, T cells expressing the αD26Y exhibited a trend towards an increase in the percentage of CD107A\(^+\) MART-1 9mer reactive T cells (Figure 36A), corresponding to the statistically significant increase in MEL 624 lysis (Figure 17).
Figure 36. Percentage of Total CD107A+ CD8+ and CD4+ MART-1 9mer Reactive T cells. The percentages of MART-1 9mer reactive (a) CD8+ and (b) CD4+ T cells expressing CD107A is displayed. Data represent the average of 6 experiments (three donors, two independent repeats) and error bars represent the standard error of the mean. As described in Chapter Two, reactivity is defined after background subtraction, specifically, any CD107A+ T cells expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). **P < 0.01; *P < 0.05 when the percentage of antigen reactive T cells given with a given modified DMF5 TCR was compared to the percentage of antigen reactive T cells expressing the WT TCR by one-way ANOVA.
Although not statistically significant compared to CD8+ T cells expressing the WT TCR, T cells expressing the αD26Y/αY50A/βL98W TCR exhibited a trend towards a decrease in the percentage of CD107A+ MART-1 9mer reactive T cells (Figure 36A) despite exhibiting comparable levels of MEL 624 lysis (Figure 17). Lastly, although not statistically significant compared to CD8+ T cells expressing the WT TCR, T cells expressing the αD26Y/αY50V/βL98W TCR or βL98W TCR exhibited comparable percentages of CD107A+ MART-1 9mer reactive T cells (Figure 36A) despite exhibiting a trend towards an increase in MEL 624 killing (Figure 17). Overall, the percentages of CD107A+ MART-1 9mer reactive CD8+ T cells roughly followed to the patterns observed in MEL 624 lysis. The one drastic exception was observed with T cells expressing the αD26Y/βL98W TCR.

TCR transduced T cells expressing either the WT DMF5 TCR or each of the mutant DMF5 TCRs used in the MEL 624 lysis assay were comprised of both CD4+ and CD8+ T cells. Therefore, we determined if the percentages of MART-1 9mer reactive CD4+ T cells expressing CD107A correlated to MEL 624 lysis (Figure 36B). Although not significant compared to CD4+ T cell expressing the WT TCR, CD4+ T cells expressing the αD26Y TCR or βL98W TCR exhibited a trend towards an increase in the percentages of CD107A+ MART-9mer reactive T cells (Figure 36B), coinciding with their significant or trend towards an increase in MEL 624 lysis (Figure 17), respectively. Compared to CD4+ T cell expressing the WT TCR, CD4+ T cells expressing the αD26Y/βL98W TCR exhibited a comparable percentage of CD107A+ MART-9mer reactive T cells (Figure 36B), despite their significant increase in MEL 624 lysis (Figure 17). Lastly, CD4+ T cells expressing the αD26Y/αY50A/βL98W TCR or
αD26Y/αY50V/βL98W TCR exhibited comparable or a trend towards an increase in the percentages of CD107A+ MART-9mer reactive T cells (Figure 36B), coinciding with their comparable or a trend towards an increase in MEL 624 lysis (Figure 17), respectively. In summary, the percentages of CD107A+ MART-1 9mer reactive CD4+ T cells corresponded to patterns in MEL 624 lysis. One exception was observed in CD4+ T cells expressing the αD26Y/βL98W TCR.

A few conclusions can be made based on the correlations between CD107A+ 9mer reactive CD4+ and CD8+ T cells and MEL 624 lysis. First, the lysis assays were done using CD4+ and CD8+ pooled T cells. As previously stated, the proportion of CD4+ T cells ranged between 27% and 35% and the proportion of CD8+ T cells ranged between 65% and 73% amongst the TCR transduced T cells populations. Therefore, we can draw overarching conclusions from the data (as we did previously), but we cannot directly compare lysis without performing the lysis assay with only CD4+ T cells or only CD8+ T cells. However, if we observe overall trends in the patterns, it is evident that the percentages of CD107A+ T cells generally correlated to the patterns observed in MEL 624 lysis. The one exception was observed with T cells expressing the αD26Y/βL98W TCR. There a few possible explanations for this result. Notably, the αD26Y/βL98W TCR harbors the supraphysiological 3D TCR affinity. It is possible that with this level of affinity, CD107A expression is not reflective of lysis. Additionally, at this level of affinity, it is possible that CD107A expression is antigen density dependent. In summary, patterns observed in CD107A expression in response to the MART-1 9mer were generally correlative with patterns observed in MEL 624 lysis, except with T cells expressing a TCR harboring a supraphysiological 3D TCR affinity. Based on all the data
observing antigen specificity and cross-reactivity in DMF5 TCR transduced CD8+ T cells expressing specific functional phenotypes, a few comprehensive conclusions were generated. First, trends in MART-1 9mer and MART-1 homolog recognition amongst T cells expressing each of the DMF5 TCRs were commonly reproducible whether examining the total percentages of antigen reactive CD8+ T cells (Figure 20), or the percentages of antigen reactive CD8+ T cells expressing a specific functional phenotype (Figures 31-35). However, these trends were not 100% reproducible with each DMF5 TCR in each individual phenotype. This suggested that observing antigen reactivity in regards to exhibiting any functional phenotype (Figure 20) gives a baseline for the total percentage of antigen reactive T cells. Subsequently, it is plausible to observe minor variation around that baseline within individual functional phenotypes (Figures 31-35). In summary, these results indicated the patterns of MART-1 and MART-1 homolog reactivity elicited with T cells expressing each of the different DMF5 TCRs are generally or broadly reproducible when comparing the total percentage of antigen reactive T cells or the percentage of antigen reactive T cells within an individual phenotype.

**Impact of Single MHC Targeted Mutations on Cross-Reactivity in DMF5 TCR**

Our findings indicated that in combination with the αD26Y and βL98W TCR mutations that target the MART-1 peptide, the addition of the αY50V mutation could reduce the cross-reactivity to levels similar to the WT TCR. Furthermore, the addition of the αY50A mutation reduced cross-reactivity lower than that of the WT TCR. We therefore determined if these mutations that weaken TCR binding to the MHC could reduce cross-reactivity while maintaining reactivity against MART-1 expressing targets.
Figure 37. Impact of Single, MHC Weakening, TCR Alpha Chain Mutations in the DMF5 TCR. Human T cells expressing either WT or mutated DMF5 TCRs were stimulated with T2 cells loaded with the control HCV NS3 peptide (KLIVALGINAV), MART-1 9mer peptide (AAGIGILTV), or MART-1 homologs. Cells were incubated with CD3/CD28 Dynabeads in a 1:1 bead to cell ratio for positive control. IFN-γ release was measured by ELISA in triplicate wells. Error bars represent the standard error of the mean from three independent repeats of one representative donor. Antigen reactivity is defined as a T cell culture that secretes twice background, or twice the amount of IFN-γ produced against T2 cells loaded with the HCV peptide, and greater than 200 pg/mL.

Mutant DMF5 TCRs were constructed with only the αY50A TCR mutation or the αY50V TCR mutation. Human T cells were transduced to express either the WT, αY50A, or αY50V DMF5 TCRs. T cells expressing the single mutant DMF5 TCRs eliminated all recognition of MART-1 and MART-1 homologs (Figure 37). Furthermore, activation with CD3/CD28 beads indicated that these T cells were capable of secreting IFN-γ. These results indicated that in the DMF5 TCR, αY50 binding to HLA-A2 is essential for antigen
recognition. However, when αY50 TCR mutations are counterbalanced with TCR mutations that enhance binding to the MART-1 peptide, the reduced αY50 TCR and HLA-A2 binding is permissible. Overall, these results exemplify the importance of this TCR/pMHC contact site for antigen recognition.

**Impact of Single MHC Targeted Mutations on Cross-Reactivity in HCV 1406 TCR**

In our structure-guided design strategy, positive TCR mutations that target the peptide would be TCR and ligand specific. However, it is plausible that the negative mutations that weaken binding with the MHC could be translatable to other TCRs if they were introduced at evolutionarily conserved residues or binding sites. The αY50 residue makes contact with an evolutionarily conserved region of HLA-A2 [37]. Therefore, we determined how the single MHC weakening mutations affected antigen recognition in another TCR. Herein, we utilized our previously described HLA-A2 restricted hepatitis C (HCV) NS3:1406-1415-reactive TCR because this TCR recognized a number of NS3 mutant epitopes [437, 438]. Mutations were made in the alpha chain to that same tyrosine that contacts the same conserved region of HLA-A2, αY59A or αY59V. A panel of naturally occurring mutant epitopes of the HCV NS3 protein were used to measure cross-reactivity [438]. Human T cells were transduced to express either the WT, αY59A, or αY59V HCV 1406 TCRs. We first determined how T cells expressing the WT HCV 1406 TCR recognized the mutant NS3 epitopes. T cells expressing the WT HCV TCR are cross-reactive against all of the mutant epitopes except 8S/9G/12L (Figure 38). After determining which mutant NS3 epitopes were recognized by T cells expressing the WT HCV 1406 TCR, we determined how the mutant HCV 1406 TCRs affected recognition of the WT and mutant NS3 epitopes.
Figure 38. Impact of Single, MHC Weakening, TCR Alpha Chain Mutations in the HCV 1406 TCR. Human T cells expressing either WT or mutated HCV 1406 TCRs were stimulated with T2 cells loaded with the control MART-1 9mer peptide (AAGIGILTV), HCV NS3 peptide (KLVALGINAV), or mutant NS3 peptides. Cells were incubated with CD3/CD28 Dynabeads in a 1:1 bead to cell ratio for positive control. IFN-γ release was measured by ELISA in triplicate wells. Error bars represent the standard error of the mean from three independent repeats of one representative donor. Black triangles indicate CD8 dependent epitopes. Antigen reactivity is defined as a T cell culture that secretes twice background, or twice the amount of IFN-γ produced against T2 cells loaded with the MART-1 peptide, and greater than 200 pg/mL.

The αY59A TCR mutation in the HCV 1406 TCR eliminated detectable all detectable recognition of the WT NS3 HCV peptide and all HCV NS3 peptide mutants. Activation with CD3/CD28 beads indicated that these T cells were capable of secreting IFN-γ. Dissimilar to the single mutations in the DMF5 TCR, the αY59V TCR mutation in the HCV 1406 TCR reduced reactivity against the WT NS3 peptide compared to the T cells expressing the WT HCV 1406 TCR. The αY59V TCR mutation in the HCV 1406 TCR reduced cross-reactivity against three mutant epitopes (V1408L, I1412L, I1412V) even
eliminating detectable cross-reactivity against four (A1409T, I1412N, V1408T, 8S/9G/12L/14S), compared to T cells expressing the WT HCV 1406 TCR. More specifically, recognition of epitopes previously determined to be CD8 dependent (A1409T, I1412N, and 8S/9G/12L/14S) were no longer recognized [438, 439]. While the 3D affinity of the DMF5 TCR is around 37 μM, the HCV 1406 TCR has a higher 3D affinity, around 16.8 μM [439]. It is possible that this increase in TCR affinity contributes to the ability of the αY59V HCV TCR to recognize the WT NS3 peptide and some of the mutant NS3 epitopes. In summary, these results indicated this conserved MHC contact residue in the TCR could be a potential site for mutation in order to reduce potential cross-reactivity of high affinity TCRs.

**Impact of Mutant DMF5 TCRs on Recognition of Processed Antigens**

Thus far, we have observed how T cells expressing each of the mutant DMF5 TCRs alter antigen recognition and cross-reactivity by recognition of T2 cells loaded with a peptide panel comprised of MART-1 homologs. However, many peptide reactive T cells do not recognize the processed antigen. Therefore, we sought to determine if the four human MART-1 homologs (human CD9, human elongation factor 1α, G-protein coupled receptor 3, and human receptor expression enhancing protein 5) were processed and presented by HLA-A2, and recognized by TCR transduced T cells expressing the WT or modified DMF5 TCRs. To answer this, we planned to transfect COS-A2 cells with cDNAs encoding the full length MART-1, human CD9, human elongation factor 1α, G-protein coupled receptor 3, and human receptor expression enhancing protein 5. However, preliminary experiments comparing COS cells, COS-A2 cells, and COS-A2 cells loaded with the MART-1 9mer revealed an unexpected result.
Figure 39. Impact of DMF5 TCRs on COS-A2 Cell Recognition. Human T cells expressing either WT or a mutant DMF5 TCR were stimulated with COS cells, COS-A2 cells, or COS-A2 cells loaded with the MART-1 9mer peptide (AAGIGILTV) for 18 hours. Antigen reactivity is as defined as a T cell culture that secretes twice background (COS cells) and greater than 200 pg/mL IFN-γ. IFN-γ release was measured by ELISA in triplicate wells. One representative experiment from is shown.

First, T cells expressing the WT DMF5 TCR did not recognize COS cells or COS-A2 cells, but did recognize MART-1 loaded COS-A2 cells (Figure 39). Surprisingly, T cells expressing the aD26Y, aD26Y/βL98W, and aD26Y/αY50V/βL98W TCRs recognized COS-A2 cells and MART-1 loaded COS-A2 cells, but not COS cells. The recognition of COS-A2 cells by T cells expressing the modified DMF5 TCRs suggested HLA-A2 restricted recognition of other antigens. This observation led us to determine if COS-A2 MART-1 recognition was unique, or if COS-A2 MART-1 recognition was due to broad cross-reactivity. Therefore, we composed a panel of tumors derived from a variety of tissues to examine potential cross-reactivity with naturally processed self-antigens (Table 2, Chapter Two - Methods).
Human T cells transduced to express the WT DMF5 TCR or each modified DMF5 TCR were used as effector T cells and stimulated with the tumor panel in cytokine release assays. Of note, included in this panel was A375, an HLA-A2^+ MART-1^- melanoma cell line. One representative donor and experiment is shown in Figure 40. First, two patterns of cross-reactivity were observed. The first pattern was mutant DMF5 TCRs that were highly cross-reactive compared to the WT TCR (generally, three times as reactive as the WT TCR) (Figure 40A). The second pattern was mutant DMF5 TCRs that were modestly cross-reactive compared to the WT TCR (generally, less than two times as reactive as the WT TCR) (Figure 40B). T cells expressing WT TCR were cross-reactive against the HLA-A2^+ off-target tumors (Figure 40A). Somewhat surprisingly, T cells expressing either the αD26Y TCR or αD26Y/βL98W TCR were reactive against all the HLA-A2^+ off-target tumors, more than T cells expressing the WT TCR (Figure 40A). Furthermore, T cells expressing the αD26Y/αY50V/βL98W TCR were reactive against all the HLA-A2^+ off-target tumors, although not as reactive as T cells expressing the αD26Y or αD26Y/βL98W TCRs, but more reactive than T cells expressing the WT TCR (except with A375) (Figure 40A). Lastly, T cells expressing the βL98W TCR or αD26Y/αY50A/βL98W TCR exhibited enhanced cross-reactivity against some, but not all of the HLA-A2^+ off-target tumors, compared to T cells expressing the WT TCR (Figure 40B). It was interesting to note that off-target tumor recognition was tumor specific within each mutant DMF5 TCR. Variability in recognition and potential off-target tumor antigens being recognized will be discussed in further detail in a subsequent section.
Figure 40. Impact of DMF5 Mutations on Cross-Reactivity Using Multi-Tissue Tumor Panel. Human T cells expressing either WT or a mutant DMF5 TCR were stimulated with tumor cell lines (see Table 2 – Chapter Two, Methods). IFN-γ release was measured by ELISA in triplicate wells. SAUJ and MEL 624-28 are HLA-A2−, all other tumors are HLA-A2+. Two patterns of off-target tumor recognition were observed. (a) Highly cross-reactive TCRs compared to the WT TCR (generally, three times as reactive as the WT TCR). (b) Modestly cross-reactive TCRs compared to the WT TCR (generally, less than two times as reactive as the WT TCR). One representative experiment and donor is shown. Reactivity is defined as a T cell culture that secretes twice background, or twice the amount of IFN-γ produced as against MEL 624-28, and greater than 200 pg/mL.
Additionally, it was surprising when T cells expressing the βL98W TCR or αD26Y/αY50A/βL98W TCR were cross-reactive, T cells expressing the αD26Y/αY50A/βL98W TCR generally exhibited higher magnitudes of reactivity. These results would not have been predicted based upon 3D affinity or our conclusions from MART-1 homolog peptide loaded targets, where T cells expressing the αD26Y/αY50A/βL98W TCR reduced cross-reactivity. In summary, T cells expressing each of the mutant DMF5 TCRs were generally more cross-reactive against the off-target, HLA-A2+ tumors than T cells expressing the WT TCR. Furthermore, the DMF5 WT TCR is cross-reactive.

Our previous results have indicated that donor variability occurs in polyclonal PBL-derived T cells. Therefore, it was of importance to analyze this off-target cross-reactivity in multiple donors. All individual experiments and donors are shown in Figures 52-58, in the appendix. In all the individual experiments, we observed variability in the patterns of recognition, magnitudes of responses, and magnitudes in background amongst T cells expressing each of the DMF5 TCRs. Thus, instead of averaging together experiments, we developed a scoring index to examine how mutations in the DMF5 TCR altered recognition of the off-target tumors compared to the WT DMF5 TCR among different donors and experiments. We based this index off of our standard of defining antigen reactivity as twice over background and greater than 200 pg/mL. Therefore, we examined each experiment and allocated a score to each mutant DMF5 TCR in response to each off-target tumor. A score of 1 indicated IFN-γ production against an off-target tumor was twice over the IFN-γ production against MEL 624-28 within the respective mutant DMF5 TCR, **AND** twice over the IFN-γ production exhibited
by the WT DMF5 TCR, AND over 200 pg/mL. A score of 0 was given if ANY of the three criteria were NOT met. This scoring was completed in each experiment in each donor. We used MEL 624-28 as the negative control because this is a relevant MART-1+ melanoma, but it is HLA-A2+. We used the WT DMF5 TCR in the scoring index so that we could compare off-target tumor recognition by each modified DMF5 TCR compared to the WT DMF5 TCR. Overall, with this scoring index we could examine both differences in recognition over background (MEL 624-28) within a respective mutant DMF5 TCR and differences in recognition compared to the WT DMF5 TCR. Table 17 specifies the scores given to each modified DMF5 TCR, in each experiment, against each off-target tumor. These scores were based on the raw data shown in Figures 52-58 in the appendix. We subsequently averaged the scores together and obtained an index that demonstrated the frequency at which T cells expressing each mutant DMF5 TCR exhibited twice the level of recognition against an off-target tumor as T cells expressing the WT TCR (Figure 41). The criteria for comparing to the WT DMF5 TCR is just classified as twice over recognition by the WT DMF5 TCR, and thus, we understand this index does not take into account the magnitude in which a response is greater than twice of the WT TCR. However, this index gives us an objective way to compare experiments given the donor and experimental variability. These results indicated T cells expressing the αD26Y TCR recognized the off-target A375, UOK131, SW480, SKGT5, and U251 tumors more than T cells expressing the WT TCR in every experiment. Furthermore, T cells expressing the αD26Y TCR recognized the off-target SKOV3, CAPAN 1, MDA 231, and HEPG2 tumors more than T cells expressing the WT TCR with a frequency between 0.83 and 0.86.
## Off-Target Tumor

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Table 17. Scoring Index for DMF5 TCRs against Off-Target Tumors. Experiment 1 in donor 1 was not included in analysis because a minimal off-target tumor panel was used. A blank space indicates off-target tumor was not used in that experiment. A score of 1 indicated IFN-γ production against an off-target tumor was twice over IFN-γ production against MEL 624-28 within the respective mutant DMF5 TCR, AND twice over IFN-γ production exhibited by the WT DMF5 TCR, AND over 200 pg/mL. A score of 0 was given if ANY of the three criteria were NOT met. Shown are scores from four donors, including 1-2 experiments, were averaged for each off-target tumor with each DMF5 TCR.
Figure 41. Frequency Mutant DMF5 TCRs Exhibited Enhanced Recognition of Off-Target Tumors Compared WT DMF5 TCR. Given experimental variability, a reactivity score was developed to enable a direct comparison of experiments. Recognition of each off-target tumor by each mutant DMF5 TCR was given a score of 1 or 0 in every experiment. A score of 1 indicated IFN-γ production was twice over IFN-γ production against MEL 624-28 (HLA-A2) within respective mutant DMF5 TCR, AND twice over IFN-γ production exhibited by the WT DMF5 TCR, AND over 200 pg/mL. A score of 0 was given if ANY of the three criteria were NOT met. Scores from each experiment were averaged for each mutant DMF5 TCR and off-target tumor. Data indicate the averages of seven experiments and error bars indicate the standard error of the mean.
T cells expressing the βL98W TCR recognized the off-target tumors more than T cells expressing the WT TCR with a frequency between 0.14 and 0.33. T cells expressing the αD26Y/βL98W TCR recognized the off-target SKOV3, MDA 231, SW480, HEPG2, SKGT5, and U251 tumors more than T cells expressing the WT TCR in every experiment. Furthermore, T cells expressing the αD26Y/βL98W TCR recognized the off-target A375, UOK131, and CAPAN 1 tumors with frequencies between 0.83 and 0.85. T cells expressing the αD26Y/αY50A/βL98W TCR did not recognize the off-target UOK131 or HEPG2 tumor more than T cells expressing the WT TCR in any experiment. Moreover, T cells expressing the αD26Y/αY50A/βL98W TCR recognized the other off-target tumors more than T cells expressing the WT TCR with frequencies between 0.14 and 0.71, with U251 yielding the highest frequency. Lastly, T cells expressing the αD26Y/αY50V/βL98W TCR recognized the off-target tumors more than T cells expressing the WT TCR with frequencies between 0.33 and 0.71. Overall, these results coincided with the two patterns of off-target recognition we previously described in Figure 40. Specifically, T cells expressing the αD26Y TCR and αD26Y/βL98W TCR exhibited greater off-target tumor recognition in nearly all the experiments compared to T cells expressing the WT TCR. T cells expressing the αD26Y/αY50V/βL98W TCR frequently exhibited greater off-target tumor recognition compared to T cells expressing the WT TCR. Lastly, T cells expressing the βL98W TCR and αD26Y/αY50A/βL98W TCR sometimes exhibited greater off-target tumor recognition compared to T cells expressing the WT TCR, but demonstrated the lowest frequencies amongst the mutant DMF5 TCRs.
Creating the previous index allowed us to examine the frequency at which T cells expressing a given mutant DMF5 TCR exhibited greater recognition of an off-target tumor than T cells expressing the WT TCR in a yes/no manner. While this provided valuable conclusions based upon all the experiments and donors, it is important to also note conclusions that were revealed based upon the magnitudes and patterns of off-target tumor recognition. First, off-target recognition was not completely reproducible among experiments. For example, in donor three, T cells expressing the αD26Y TCR exhibited comparable recognition of SKOV3 (3, 128 pg/mL) and UOK131 (3,671 pg/mL) in experiment one (Figure 55, in the appendix), but were 4.5 times more reactive against UOK131 (17,469 pg/mL) than SKOV3 (3,854 pg/mL) in experiment two (Figure 56, in the appendix). Secondly, off-target recognition was donor dependent. For example, T cells expressing the αD26Y/αY50V/βL98W exhibited similar levels of recognition of the off-target U251 tumor compared to T cells expressing the WT TCR in donor two (Figures 53-54, in the appendix) but increased recognition in donor four (Figures 57-58, in the appendix). Thirdly, T cells expressing the WT DMF5 TCR recognized at least one off-target tumor in every experiment. This occurred against MDA 231 and CAPAN 1 (Figure 52, in the appendix), CAPAN 1 (Figure 53, in the appendix), CAPAN 1 and SW480 (Figure 54, in the appendix), CAPAN 1 (Figure 55, in the appendix), A375, SCOV 3, UOK131, CAPAN 1, MDA 231, and SW480 (Figure 56, in the appendix), A375, SKOV3, CAPAN 1, and MDA 231 (Figure 57, in the appendix), and A375, SKOV3, UOK131, CAPAN 1, MDA 231, SW480, HEPG2, SKGT5, and U251 (Figure 58, in the appendix). Specifically, T cells expressing the WT TCR recognized the CAPAN 1 tumor in every experiment. Conclusively, all of these results suggested
off-target tumor recognition was generally experiment and donor dependent. Despite
donor and experimental variability, a few overarching conclusions in regards to off-
target tumor recognition can be made. First, the observed cross-reactivity was HLA-A2
restricted. Secondly, T cells expressing the WT DMF5 TCR are cross-reactive. Thirdly,
T cells expressing the αD26Y TCR or αD26Y/βL98W TCR are highly cross-reactive
against all the tumors. T cells expressing the αD26Y/αY50V/βL98W TCR are frequently
cross-reactive against all the tumors. T cells expressing the βL98W TCR are mildly
cross-reactive against the tumors and sometimes this recognition is above the level of
recognition by T cells expressing the WT TCR. Lastly, T cells expressing the
αD26Y/αY50A/βL98W TCR are mildly cross-reactive against the tumors and sometimes
this recognition is above the level of recognition by T cells expressing the WT TCR or
βL98W TCR.

**Impact of Mutant DMF5 TCRs on 2D Affinity**

It was very interesting that the patterns of cross-reactivity against the HLA-A2+
tumors did not coincide with the DMF5 TCRs measured 3D affinity. More recently, it has
been demonstrated that 2D affinity measurements can better predict T cell functional
outcomes compared to 3D affinity measurements [162, 170, 172]. This is most likely
due to the fact that 2D affinity measurements account for aspects unique to membrane-
bound proteins, where 3D affinity measurements are strictly dependent upon the
TCR/pMHC. We measured the 2D affinity of the WT DMF5 TCR and each of the mutant
DMF5 TCRs to determine if these measurements correlated to the observed patterns in
off-target tumor reactivity. To eliminate the variability associated with polyclonal PBL-
derived T cells, we utilized Jurkat E6.1 cells transduced with the WT DMF5 TCR or
each of the mutant DMF5 TCRs for this experiment. The 2D affinity measurements of
the DMF5 TCRs with the MART-1 10mer peptide/HLA-A2 complex are shown in Figure 42. The observed patterns of cross-reactivity against the off-target tumor panel better correlated with 2D affinity measurements. Specifically, T cells expressing the αD26Y TCR or the αD26Y/βL98W TCR exhibited the highest 2D affinity, corresponding to their high levels of cross-reactivity against the off-target tumors. Most notably, the αD26Y/αY50A/βL98W TCR and αD26Y/αY50V/βL98W TCR revealed higher affinities compared to the βL98W or WT TCR, which coincided with their enhanced cross-reactivity against the off-target tumor lines. One explanation for the disconnect between 3D and 2D affinity measurements could be due to differences in TCR affinity when using purified proteins or membrane-bound interactions. In conclusion, 2D affinity measurements correlated better to the cross-reactivity observed against the off-target tumor panel than the 3D affinity measurements.

**Potential DMF5 TCR Targets in Off-Target Tumors**

In was interesting that the patterns of recognition against the MART-1 homologs and off-target tumors were not consistent amongst each of the different DMF5 TCRs. Thus, we began to hypothesize what targets these TCRs were recognizing on the off-target tumor cells. First, we knew that they recognized specific peptides, or a class of peptides in the context of HLA-A2, because T2 cells loaded with negative control peptides and some of the MART-1 homolog peptides revealed no detectable reactivity. We refocused our attention on the human self-peptides, MART-1, human CD9, human elongation factor 1α, G-protein coupled receptor 3, and human receptor expression enhancing protein 5. We initially hypothesized it was possible that the T cells were recognizing one or more of these proteins on the off-target tumor cells.
Figure 42. Relative 2D Affinity of DMF5 TCRs. Adhesion frequencies of TCR transduced Jurkat E6.1 cells were determined using a two-dimensional micropipette adhesion frequency assay with TCR transduced Jurkat E6.1 cells expressing each DMF5 TCR and MART-1 10mer/HLA-A2-coated RBCs. Relative 2D affinities were calculated using the specific adhesion frequency (Pa) along with the surface pMHC (m$_1$) and TCRβ (m$_2$) densities, as determined by flow cytometry. For each TCR, 50 Jurkat E6.1 cell-RBC pairs were used to obtain P$_a$ values from which the affinity was calculated as: A$_c$K$_a$ = −ln [1−P$_a$(1)]/m$_1$m$_2$. The geometric mean of affinities and normalized adhesion bonds are reported ± standard error of the mean. 2D affinity measurements were completed in the Evavold lab.
We utilized “The Human Protein Atlas” to determine if these proteins were expressed in the tissues from which our off-target tumors were derived. Aside from MART-1 expression being limited to only melanocytes, the other four self-proteins were expressed either at high, medium, or low levels in a majority of tissues throughout the human body (Table 18). One caveat here is that we do not know the expression of the proteins in our specific tumors. However, these results implicated there was a possibility that some of these self-proteins could be expressed in the tumors. Since human CD9 is expressed on the cell surface, we immunofluorescently labeled the tumors with an anti-CD9 mAb to determine if CD9 was expressed on the tumor cells. We confirmed expression of CD9 on all the tumor cell lines (Figure 43). Specifically, human CD9 expression on the tumors ranged between 44% and 100%. Human elongation factor 1α, G-protein coupled receptor 3, and human receptor expression enhancing protein 5 are not expressed on the surface and thus, could not be immunofluorescently labeled. Other assays such as RT-PCR could confirm mRNA transcripts or western blot could confirm protein expression. However, once we knew the off-target tumors were CD9+, expression of one of more of the other three self-proteins would still not directly elucidate what antigens were being recognized. Although it was that plausible that CD9 was being recognized on the off-target tumor cells by the DMF5 TCR transduced T cells, we believed we had thorough evidence to suggest the cross-reactivity was due to multiple different antigens, and was most likely DMF5 TCR dependent. Variability in the recognition of the off-target tumors between DMF5 TCRs, donors, and experiments indicated a few possibilities in regards to the antigens being recognized.
Table 18. Levels of MART-1 and Self-MART-1 Homolog Protein Expression in Normal Tissues. “X” indicates not expressed. These data were generated from The Human Protein Atlas.

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Figure 43. Human CD9 Expression on Tumor Panel. 1x10^6 tumor cells were stained for surface expression of HLA-A2 and CD9. (a) PG13 cells (murine leukemia cell line) were used as a negative control. SAUJ tumor cells show an example of an HLA-A2^-CD9^+ tumor line while A375 tumor cells (MART-1) show an example of an HLA-A2^+CD9^+ tumor line. (b) Summary of surface CD9 expression on tumor cell lines. Data represent the averages of two independent experiments and error bars indicate the standard error of the mean.
The highly cross-reactive TCRs (αD26Y TCR and αD26Y/βL98W TCR) could be eliciting low levels of cross-reactivity to numerous antigens being broadly recognized, or could be eliciting high levels of cross-reactivity to one or a few antigens. Variability in recognition of the different off-target tumors by even the same DMF5 TCR transduced T cell population indicated is it unlikely the off-target tumors are processing and presenting the same antigen or antigens. Furthermore, it is possible the DMF5 TCRs are recognizing the same set of antigens on the off-target tumor cells, just at different levels of magnitudes. Conversely, it is possible the DMF5 TCRs are each recognizing a completely different spectrum of antigens. In summary, it is possible that human CD9 was recognized on the off-target tumor cells, but we believe this was not the sole potentially recognized antigen.

We believed our data thus far indicated that T cells expressing each of the mutated DMF5 TCRs were recognizing multiple different peptides on the off-target tumors. Therefore, it was of interest to expand the number of targets, but also categorize the targets we were examining in order to narrow down potential targets. We elucidated potential targets by utilizing combinatorial peptide libraries in positional scanning format [440, 441]. In combinatorial peptide libraries in positional scanning format, each pool contains a mixture of peptides where one or more of the peptide residues’ is fixed at a specific amino acid and the remaining peptide residues include a mixture of amino acids. This method allowed us to examine reactivity against millions of peptides and highlight favorable amino acids at specific sites in the peptide for recognition. In this analysis we utilized TCR transduced Jurkat 76 cells to limit the donor variability observed in polyclonal T cell populations. The Jurkat 76 cell line is TCR
and therefore, we eliminated off-target recognition due to TCR chain mispairing in using this cell line. For this analysis, we compared Jurkat 76 cells expressing the WT DMF5 TCR with Jurkat 76 cells expressing the highly cross-reactive αD26Y/βL98W TCR, or Jurkat 76 cells expressing the unexpectedly, off-target tumor cross-reactive, αD26Y/αY50A/βL98W TCR (Figure 4) [350]. Jurkat 76 cells expressing the WT DMF5 TCR exhibit recognition of nearly all the sub-libraries, supporting the notion that this WT DMF5 TCR is inherently cross-reactive. Jurkat 76 cells expressing the αD26Y/βL98W TCR exhibited enhanced recognition against the sub-libraries compared to the WT DMF5 TCR expressing Jurkat 76 cells. Lastly, Jurkat 76 cells expressing the αD26Y/αY50A/βL98W TCR exhibited a reduction in recognition against the sub-libraries compared to the WT DMF5 TCR expressing Jurkat 76 cells. Overall, these data support our conclusions based on the MART-1 homolog peptide data, where the αD26Y/βL98W TCR enhanced cross-reactivity and the αD26Y/αY50A/βL98W TCR reduced cross-reactivity compared to the WT TCR.

This approach of using a combinatorial peptide library, has been utilized to identify recognition of an off-target, altered-peptide ligand of an antigen specific TCR [442]. Albeit, in our model it would be difficult to identify important residues at specific peptide positions for antigen recognition because recognition generally occurred across all peptide pools with the WT TCR and αD26Y/βL98W TCR. However, the αD26Y/αY50A/βL98W TCR largely reduced Jurkat 76 cell potency against the peptide pools compared to the WT TCR and displayed a less permissive pattern of reactivity against the peptide pools. One notable difference was observed in the recognition of the peptide pool containing a fixed aspartic acid at position 5.
**Figure 4. Combinatorial Peptide Library Scans.** Combinatorial peptide library scans of WT (top), αD26Y/βL98W (middle), and αD26Y/αY50A/βL98W (bottom) TCRs. For each experiment, TCR transduced Jurkat 76 cells were incubated with equal numbers of T2 cells loaded with 152 sub-libraries where each position of the peptide (excluding primary anchors) was fixed with each of the naturally occurring amino acids (excluding cysteine). Reactivity was assessed by measuring IL-2 release in triplicate wells, as indicated by the heat map scales on the right. Results are the average of three independent experiments with each TCR. This experiment was completed in the Baker lab [350].
Reactivity was about doubled with cells expressing the αD26Y/αY50A/βL98W TCR compared to Jurkat 76 cells expressing the WT DMF5 TCR. This suggested that despite generally reducing overall cross-reactivity compared to the WT DMF5 TCR, there were possibly a small number of peptides that elicited an increase in reactivity with Jurkat 76 cells expressing the αD26Y/αY50A/βL98W TCR. This idea would support our findings as to why T cells expressing the αD26Y/αY50A/βL98W TCR were sometimes more cross-reactive against the off-target tumors than T cells expressing the WT DMF5 TCR. This pool (fixed aspartic acid at position 5) is also notable because it was demonstrated that the WT DMF5 TCR also recognizes epitopes containing a “DRG” charged core [404, 423]. In summary, the potential number of targets recognized on the tumor cells is seemingly vast, however, recognition via the αD26Y/αY50A/βL98W TCR could be the result of enhanced reactivity against a few targets compared to the WT TCR.

In summary, in collaboration with the Baker’s laboratory, we have developed a novel structure guided approach to enhance antigen specificity and reduce cross-reactivity. We generated five modified DMF5 TCRs and sought to determine how structure-guided mutations in the DMF5 TCR altered tumor lysis, polyfunctional T cell responses, cross-reactivity, antigen specificity, and recognition of processed antigens. When measuring polyfunctional T cell responses against a panel of MART-1 homologs, the structure guided approach appeared to be promising, with T cells expressing the αD26Y/αY50A/βL98W TCR exhibiting reduced percentages of MART-1 homolog reactive T cells compared to T cells expressing the WT TCR (Figure 20). Conversely, when observing T cell responses against processed antigens on a variety of different tissues, this trend did not remain consistent (Figure 40). Our results demonstrated that
sequence homology is not the sole factor in cross-reactivity, and 3D affinity against the “cognate” antigen is not always correlative to all-encompassing cross-reactivity. Furthermore, T cell responses vary due to biophysical properties/kinetics of the TCR/pMHC interaction and the polyclonal PBL-derived T cell population. Our structure-based designed strategy could be utilized in the future, as it is more meticulous and specific than random mutation through yeast or phage display. Specifically, we also elucidated the critical role of the MHC weakening TCR mutation and how it could potentially be translatable to other TCRs. Overall, it is important to understand how alterations in the TCR/pMHC interface can affect functional T cell phenotypes to maximize the efficacy and safety of TCRs to be used in gene modified T cells in adoptive cell transfer.

Second Round of DMF5 TCR Mutations

The goal of designing the first round of DMF5 mutations (DMF5 TCRs described thus far) was to determine which TCR mutations enhanced MART-1 specificity while reducing off-target cross-reactivity. Our objective was to take the information we learned from the first generation of mutant DMF5 TCRs and subsequently optimize the results through the generation of a second round of mutant DMF5 TCRs. Based on information elucidated from the functional results, the Baker lab subsequently designed a second round of DMF5 TCRs. In αD26Y/αY50A/βL98W TCR expressing CD8+ T cells, a reduction in the percentage of MART-1 homolog reactive T cells was observed compared to T cells expressing the WT TCR, but a reduction in MART-1 potency was also observed. The objective with this next round of DMF5 TCRs was to better maintain MART-1 potency while also maintaining a reduction in cross-reactivity compared to the
WT TCR, similar to the level observed with the αD26Y/αY50A/βL98W TCR against the MART-1 homologs. Our results indicated the αY50 residue was critical for antigen recognition. Specifically, the alanine mutation was more effective than the valine mutation at reducing MART-1 reactivity and cross-reactivity. In collaboration with the Baker lab, we hypothesized the αY50 residue was too close to the MART-1 peptide (about 5 Å away). Therefore, the mutation of a tyrosine to an alanine at position 50 in the TCR alpha chain weakened bonds with the MHC, but also likely weakened bonds with the MART-1 peptide. Specifically, it is possible that the αY50A TCR mutation may not be solely “MHC binding” specific. This could be an explanation for the reduction observed in MART-1 specific reactivity. Thus, we wanted to examine other TCR mutations at different TCR/MHC binding residues that might not interfere with MART-1/TCR binding interactions. The Baker lab identified four residues in the DMF5 TCR that interacted with HLA-A2, but were more than 10 Å away from the MART-1 peptide. These residues included αN52, αK68, βN52, and βT57 [443]. Each residue was mutated to an alanine because the alanine mutation in the αY50 mutations was more effective than the valine mutation. Four DMF5 TCRs were made: αD26Y/αN52A/βL98W, αD26Y/βN52A/βL98W, αD26Y/αK68A/βL98W, and αD26Y/βT57A/βL98W. Each new negative mutation replaced the previous αY50 mutation. The second round of DMF5 TCRs was further used in functional T cell assays.

The objective was to determine how this second round of DMF5 mutations altered MART-1 and MART-1 homolog reactivity compared to the WT TCR. However, findings from the first round of DMF5 TCR mutations demonstrated unexpected off-
target tumor cross-reactivity that did not correlate with recognition of the MART-1 homologs. Thus, we believed we should first examine recognition of our tumor panel before examining polyfunctional T cell responses. Our preliminary experiment in one donor is shown in Figure 59, in the appendix. T cells expressing the WT DMF5 TCR are cross-reactive against HLA-A2\(^+\) tumors as previously observed. We next determined how T cells expressing the newly designed mutant DMF5 TCRs altered the recognition of the off-target tumors compared to the WT TCR. T cells expressing each mutant DMF5 TCR exhibited more cross-reactivity against all the HLA-A2\(^+\) tumors compared to T cells expressing the WT TCR. The \(\alpha\)D26Y/\(\alpha\)N52A/\(\beta\)L98W, \(\alpha\)D26Y/\(\beta\)N52A/\(\beta\)L98W, and \(\alpha\)D26Y/\(\beta\)T57A/\(\beta\)L98W TCR expressing T cells were more reactive against MEL 624 compared to T cells expressing the WT TCR, but were extremely more cross-reactive. Our previous findings indicated the presence of the \(\alpha\)D26Y mutation in a mutant TCR enhanced cross-reactivity against the off-target tumors amongst all the DMF5 TCRs. It is possible that same phenomenon is occurring with these TCR mutations as well. These mutant DMF5 TCRs appear to be extremely cross reactive compared to the WT TCR, whereas the \(\alpha\)D26Y/\(\alpha\)Y50A/\(\beta\)L98W TCR was mildly cross-reactive compared to the WT TCR. This indicated it was possible that the proximity of the \(\alpha\)Y50A mutation with the peptide was important to counterbalance the effects of the \(\alpha\)D26Y mutation. Thus, these new negative mutations do not counterbalance the effect of the \(\alpha\)D26Y mutation as efficiently. Overall, these results indicate this second round of TCR mutations did not reduce the cross-reactivity of the WT TCR.
In vivo Model

All of the experiments thus far have examined antigen specificity and cross-reactivity in vitro. It was important to next determine if these results were reproducible in vivo. However, in vitro findings do not always translate in vivo [444]. Therefore, we wanted to determine if the first round of DMF5 mutations could have anti-tumor activity in a murine model. Namely, we wanted to determine if the high affinity (3D) TCRs (αD26Y and αD26Y/βL98W) could control tumor growth better than the WT TCR and if the low affinity (3D) TCR (αD26Y/αY50A/βL98W) could control tumor growth. We chose to use a NSG A2+ mouse model for multiple reasons. First, an immunodeficient host allowed us to use a xenograft model. This was beneficial for examining the efficiency of human TCR transduced T cells against a human tumor in vivo [384, 445]. Furthermore, HLA-A2+ mice allowed us to examine any signs of autoimmunity due to the introduction of the TCR transduced T cells. Mice do not share complete homology with humans, however, three of the four self-MART-1 homologs in the peptide panel (CD9, elongation factor 1α, and receptor expression enhancing protein 5) are completely homologous in the mouse. In summary, we believed the NSG A2+ mice were an advantageous model to use to examine the anti-tumor efficacy of TCR transduced T cells and observe potential autoimmunity.

Despite the advantages of the xenograft model, there are a few drawbacks. First, due to the lack of a host immune system, there is no cytokine or chemokine support to assist in T cell function, persistence, and trafficking to the tumor [446-448]. Furthermore, in our hands and in other studies, human T cells expressing the DMF5 TCR fail to control tumor growth in NSG mice [449]. Therefore, we explored additional mechanisms
to support the persistence, function and trafficking of the introduced TCR transduced T cells. Herein, those mechanisms included prior PBMC engraftment, cytokine support, and checkpoint blockade.

The immunodeficiency of NSG mice is advantageous for xenograft models but disadvantageous for the support of the introduced human T cells. To overcome this, previous studies have generated humanized NSG mice in order to reconstitute a human immune system in the mice and support introduced T cells [450]. Thus, xenografts could be examined in the presence of a human immune system. This has been commonly done by engrafting human bone marrow-derived or umbilical cord blood-derived CD34+ hematopoietic stem cells into the mice [450-453]. We believed we could recapitulate this via the engraftment of human PBMCs. We first determined if human PBMCs could persist weeks after engraftment and determined if intraperitoneal or retro-orbital route of injection affected engraftment (Figure 60, in the appendix). These results indicated human CD3+ T cells could be detected in the blood of mice 72 days post engraftment. Furthermore, retro-orbital route of injection persisted better than the intraperitoneal route of injection, meaning the intravenous route of injection was superior for engraftment. Consistent with previous studies, mice demonstrated signs of GVHD around nine weeks post engraftment, indicating xeno-cross-reactivity occurred. In summary, these results indicated human T cells can persist 72 days post engraftment in the NSG A2+ mice.

We confirmed engrafted human T cells could persist in the blood of NSG A2+ mice and retro-orbital route of injection was superior to intraperitoneal route of injection (Figure 60, in the appendix). We next determined if the engrafted T cells could support
the persistence and function of the introduced TCR transduced T cells. Mice were engrafted with human PBMC 7 days prior to MEL 624 tumor challenge (Figure 61, in the appendix). Mice were treated with TCR transduced T cells 17 days post tumor challenge. For this pilot experiment, we only used T cells transduced with either the WT TCR, the αD26Y TCR, or the αD26Y/αY50A/βL98W TCR. These results indicated that the engraftment prior to tumor challenge did not affect the anti-tumor activity of the TCR transduced T cells, as there were no differences between treated and untreated mice. Additionally, no signs of autoimmunity were observed in these mice throughout the duration of this experiment. This indicated that the introduced T cells did not elicit off-tumor off-target damage that could be observed. To determine if engraftment supported the introduced T cells, we monitored the persistence of the TCR transduced T cells post injection. It was interesting that on day 13, no CD34+ T cells were found in the blood of treated mice, despite 66-76% CD34 expression amongst the TCR transduced T cell groups prior to injection (Figure 62, in the appendix). Specifically, in one mouse, only 0.57% of cells in blood were CD3+CD34+. There was a large population of human CD3+ T cells in the blood, however, we are unable to distinguish engrafted T cells from T cells that have lost the CD34 transgene expression. Transgene expression has been shown to decrease over time in vivo but it has been shown that cytokines can help maintain transgene expression [275, 454]. Because the TCR transduced T cells are cultured ex vivo in IL-2 and IL-15, then enter the in vivo environment with no cytokine support, we hypothesized additional cytokine support would enhance the transgene expression and T cell function.
IL-2 and IL-15 are important for T cell activation, proliferation, and function [455-460]. Furthermore, IL-2 and IL-15 have been shown to promote long-term survival and function of adoptively transferred T cells in vivo [461-466]. We therefore determined if IL-2 or IL-15 support would enhance the anti-tumor activity of TCR transduced T cells in vivo (Figure 63, in the appendix). In this pilot experiment, we compared T cells expressing the WT DMF5 TCR with untransduced T cells, with or without IL-2 or IL-15 cytokine support. There were no differences between treatments with untransduced and WT TCR transduced T cells, with or without cytokine support. These results indicated that cytokine support did not enhance the anti-tumor activity of the TCR transduced T cells. Furthermore, on day 10 post therapy, there were no differences in the percentages of CD3⁺CD34⁺ human T cells in the blood of mice treated with IL-2 or IL-15 (Figure 64, in the appendix). One mouse treated with WT TCR transduced T cells actually exhibited 1.88% CD3⁺CD34⁺ human T cells in the blood compared to 0.39% and 0.49% of IL-2 and IL-15 treated mice, respectively. These results indicated that cytokine support did not enhance transgene expression in vivo. One explanation for this could be that the TCR transduced T cells did not traffic to the tumor. To examine this, on day 16 post treatment, we determined if there were TCR transduced T cells in the tumor (Figure 65, in the appendix). Compared to untransduced T cells, there were no CD3⁺CD34⁺ T cells found in the tumors of mice treated with DMF5 WT TCR transduced T cells with or without IL-2 and IL-15. These results indicated that cytokine support did not enhance the trafficking of TCR transduced T cells to the tumor or the survival of TCR transduced T cell in the tumor. On day 16, we also determined if the TCR transduced T cells had homed and remained in the spleen (Figure 66, in the appendix).
No human CD3+ or human CD3+CD34+ T cells were found in the spleens of mice from each treatment group. Conclusively, our results suggested the introduced TCR transduced T cells were unable to survive *in vivo* despite IL-2 or IL-15 cytokine support.

Our results thus far have demonstrated that engraftment of human T cells prior to treatment or cytokine support did not enhance the anti-tumor efficacy, persistence, or trafficking of TCR transduced T cells *in vivo*. The TCR transduced T cells underwent *ex vivo* activation for up to three weeks prior to injection *in vivo* and thus, exhaustion could be limiting their effector function *in vivo* [467-469]. Therefore, we determined if anti-PD-1 treatment with adoptively TCR transduced T cells could enhance their anti-tumor efficacy [470, 471]. In this experiment, we compared the anti-tumor efficacy of untransduced or WT DMF5 TCR transduced T cells with or without anti-PD-1 (Figure 67, in the appendix). These results indicated that treatment with anti-PD-1 did not enhance the anti-tumor efficacy of WT DMF5 TCR transduced T cells, as they did not control tumor growth better than no treatment or untransduced T cells. In summary, anti-PD-1 treatment did not enhance the anti-tumor efficacy of DMF5 TCR transduced T cells *in vivo*.

Our *in vivo* data thus far suggested that TCR transduced T cells did not control MEL 624 tumor growth and were not found in the tumor after treatment. Furthermore, anti-PD-1 treatment did not affect the anti-tumor efficacy of the TCR transduced T cells, indicating that PD-1 dependent exhaustion was most likely not the sole reason. Conclusively, these results suggested loss of transgene expression and inability to traffic to the tumor potentially limited the TCR transduced T cells anti-tumor efficacy. To determine if inability of trafficking to the tumor was contributing to this, we performed an
in vivo cytotoxic T lymphocyte (CTL) assay [472]. This assay allowed us to determine if TCR transduced T cells could kill MART+ target cells in vivo without needing to traffic to the site of a tumor. A schematic of this assay is depicted in Figure 68, in the appendix. If DMF5 TCR transduced T cells killed MART-1 9mer pulsed PBMCs in vivo, we would expect to see an increase in the proportion of CFSE low (PBMCs) cells because the CFSE high cells (MART-1 9mer pulsed PBMCs) were killed. An example of the proportion of CFSE high and CFSE low cells from the spleen two days after injection is shown in Figure 69, in the appendix. In PBS treated mice, the proportion of CFSE high to CFSE lows cells remains even, at 7.04% high and 6.83% low, indicating no specific lysis occurred. In mice treated with WT DMF5 TCR transduced T cells, the proportion of CFSE high to CFSE low cells is skewed, at 11.8% low and 6.49% high. These results indicated that MART-1 9mer pulsed PBMCs (CFSE high) were killed. The % MART-1 lysis is shown in Figure 70, in the appendix. These results indicated that WT DMF5 TCR transduced T cells killed about 41% of the MART+ target cells, compared to 5% with PBS treatment. These results suggested WT DMF5 TCR transduced T cells can kill MART+ targets in vivo. Since these T cells did not have to traffic to encounter these MART+ target cells in vivo, it is plausible that we previously did not observe anti-tumor activity of the TCR transduced T cells due to their inability to traffic to the site of the tumor or suppressive mechanisms in the tumor microenvironment.
CHAPTER FOUR

DISCUSSION

Introduction

The use of TCR gene-modified T cells for adoptive T cell transfer is an evolving and promising form of immunotherapy. However, the use of TCRs that target self-antigens involve the caveat of bearing a low affinity for the targeted antigen. TCR affinity enhancement via methods such as yeast or phage display have been utilized to enhance anti-tumor efficacy of TCR transduced T cells, but have resulted in patient fatalities [5, 6, 298, 299]. Therefore, reducing potential off-target cross-reactivity while maintaining or enhancing the anti-tumor activity of TCR gene-modified T cells is of upmost importance for both safety and efficacy. Herein, we developed a novel structure-guided approach designed to fine-tune the antigen specificities of the DMF5 TCR.

Numerous factors can impact the functional response of T cells. These factors include structural components in the TCR/pMHC interface, kinetic factors, and cellular factors. In this dissertation we focused on how these factors influence the on-target and off-target responses of T cells engineered to express the WT or mutated DMF5 TCRs. Furthermore, our findings emphasize the importance of rigorous preclinical testing of modified TCRs and the need for advancement in modeling/prediction tools for protein interactions. Overall, this is important in order to better design TCRs that will be safe and more efficient in patients.
How DMF5 Mutations Affect Recognition of MART-1 Homologs

Correlation to Alanine Scan

The alanine scan method was utilized to determine that Titin was the target of the affinity enhanced MAGE-A3 TCR, causing lethal adverse events. Two sets of alanine substituted or glycine substituted peptide panels elucidated critical residues for recognition of MAGE-A3 by T cells expressing the affinity enhanced TCR. Searches for protein sequences with epitope homology resulted in three clinically relevant potential targets aside from MAGE: one was Titin, two were pathogenic [299]. Overall, this method was suitable for predicting a critical cross-reactive peptide with this modified TCR, and if completed prior to therapeutic treatments, the authors could have identified the target that led to lethal adverse events.

We utilized a panel of MART-1 nonameric alanine substituted peptides to determine how mutations in the DMF5 TCR affected recognition of a structurally altered MART-1 peptide. The alanine scan method can be a suitable method for making a general prediction about the importance of an individual residue in the peptide for recognition, and furthermore, it was an adequate tool for predicting the lethal off-target peptide in the MAGE-A3 clinical trial. However, our results indicated that this method is not always an adequate predictor for potential off-target, altered-peptide ligand reactivity. Since the alanine scan data were generated in TCR transduced CD8+ Jurkat E6.1 cells, the results must be compared with the reactivity of TCR transduced CD4+ T cells. Specifically, the alanine scan demonstrated the importance of positions 4 and 6 in the MART-1 9mer peptide for recognition by αD26Y TCR expressing cells. However, αD26Y TCR expressing CD4+ T cells recognized all of the seven MART-1 homologs
that did not display sequence homology with the MART-1 9mer peptide at position 4 and/or position 6. The alanine scan is a low throughput screen that only allows for determination of a residue’s importance for TCR recognition when mutated to an alanine, and when mutated individually. Therefore, this method lacks the ability to account for the effects of non-alanine mutations in the peptide and the effects of multiple mutations in the peptide.

It has been shown that the conformational adaptability of TCRs is important for the ability to recognize multiple different ligands [473, 474]. As a result, recognition of a peptide is more dependent upon the net effect of all the individual residues and the TCR’s ability to conform to that pMHC structure. For instance, one amino acid substitution could result in an unfavorable interaction with the TCR, but another amino acid substitution elsewhere, in the same peptide, could result in a favorable interaction with the TCR. Therefore, different interactions between the peptide and the MHC and between the peptide and the TCR can offset each other differently. These interactions will be both ligand and TCR dependent. In summary, recognition of the alanine substituted MART-1 peptides did not entirely predict recognition of different MART-1 homologs because each amino acid in a given peptide will contribute differently to binding in the MHC and to binding with the TCR to subsequently affect T cell activation and T cell function.

**Correlation to Structure**

Our results indicated that the structural effects of the introduced DMF5 TCR mutations did not always correlate to the predicted recognition of MART-1 homologs. The αD26Y TCR mutation was designed to enhance charge complementarity with the
N-terminal region of the MART-1 peptide, as previously described in Chapter Three [350, 351]. However, this TCR mutation also eliminated charge repulsion with a negatively charged glutamic acid in the HLA-A2 α1 helix [349]. This illustrates the difficulty associated with identifying TCR residues that will only impact binding with the peptide when mutated to a different amino acid. With the αD26Y TCR mutation, it is not easy to distinguish the influence of the favorable HLA-A2 binding interaction with the influence of the favorable peptide binding interaction. It is plausible that both factors were contributing to the enhanced cross-reactivity.

Our data suggested that the tyrosine mutation at position 26 in the TCR alpha chain non-specifically enhanced recognition of the MART-1 homologs. We will again focus on the CD4+ T cells for this part of the discussion because the structure-guided mutations were designed in the absence of CD8. Although not statistically significant, the percentages of MART-1 9mer and 10mer reactive T cells was modestly enhanced with T cells expressing the αD26Y TCR vs. the WT TCR, as predicted (Figure 19). The percent reactive CD4+ T cells was enhanced against every MART-1 homolog (reaching statistical significance in two) in αD26Y expressing T cells compared WT expressing T cells (Figure 19). The replacement of a charged aspartic acid with a bulky and amphipathic tyrosine enhances surface area and allows for increased involvement in van der Waals forces [395]. Therefore, the αD26Y TCR mutation seemingly enhanced non-specific binding to nonpolar, hydrophobic residues at position 2 in the 9mer peptides. In the MART-1 homologs, all residues at position two are a hydrophobic amino acid, except for in one MART-1 homolog. In ADP-ribose diphosphatase there is a charged aspartic acid at position two. ADP-ribose diphosphatase was minimally
recognized by T cells expressing the WT TCR (1.67%) but there was a stark increase in
the percentage of αD26Y TCR expressing reactive T cells (16.47%). This result was
most likely due to the elimination of the charge repulsion between the negatively
charged aspartic acids associated with the WT DMF5 TCR and ADP-ribose
diphosphatase by the tyrosine TCR alpha chain mutation. There is no clear importance
or ranking of preferred hydrophobic residues at position two in the peptide with the
αD26Y TCR. This suggested that the tyrosine mutation enhanced non-specific binding.
Overall, the αD26Y TCR mutation resulted in non-specific cross-reactivity with all the
MART-1 homologs, indicating its lack of specificity for the MART-1 peptide.

The second MART-1 targeting TCR mutation, βL98W, was designed to enhance
shape complementarity with the leucine at position 7 in the MART-1 9mer peptide, as
previously described in Chapter Three [350, 351]. Although not statistically significant,
the percentages of MART-1 9mer and 10mer reactive CD4+ T cells was modestly
enhanced with T cells expressing the βL98W TCR vs. the WT TCR, as predicted
(Figure 19). There were only minor increases observed (averaging between 0.64% and
4.3%) in the percentages of MART-1 homolog reactive CD4+ T cells between the WT
TCR and βL98W TCR, with none reaching statistical significance (Figure 19). Although
the βL98W TCR mutation enhanced binding to the MART-1 9mer via the leucine at
position 7, increased percentages of reactive βL98W TCR expressing CD4+ T cells
were only notable against one out of the four MART-1 homologs that contained a
leucine at position 7, ADP-ribose diphosphatase (5.9% vs. 1.6%) (Figure 19). This could
also be due to the valine N-terminal anchor in this peptide, as valines are superior HLA-
A2 anchors [404]. Like tyrosine, tryptophan is also commonly used to enhance binding
due to their rigid, bulky, and amphipathic nature [72]. Position 7 in other MART-1 homologs is generally also very hydrophobic, and thus the tryptophan mutation seemingly remains unable to discern differences between the hydrophobic residues. There is also no pattern in preference for specific residues at position 7 in the peptide with the βL98W TCR. In conclusion, the tryptophan TCR mutation at position in 98 in the TCR beta chain was not entirely specific for the MART-1 peptide, but was more specific than the αD26Y TCR mutation.

The field generally considers high affinity TCRs are optimal for T cell function and therapeutic efficacy [180, 396]. However, our results did not entirely correlate with this assumption when combining the αD26Y TCR and βL98W TCR mutations. The correlation with affinity will be discussed in the next section. CD4+ T cells expressing the αD26Y/βL98W TCR starkly reduced the percentage of MART-1 9mer and 10mer reactive T cells compared to T cells expressing the WT TCR (Figure 19). This results indicated that the combination of the two MART-1 targeting mutations was not additive, although affinity is dramatically increased. T cells expressing the αD26Y/βL98W TCR exhibited in a trend towards an increase in the percentages of MART-1 homolog reactive T cells compared to T cells expressing the WT TCR, with statistical significance reached with two MART-1 homologs (Figure 19). We would have expected to see more considerable differences in the recognition of the MART-1 homologs, based upon the recognition of the MART-1 homologs by T cells expressing the αD26Y TCR. Therefore, these results were most likely due to the supraphysiological high affinity of the αD26Y/βL98W TCR. This will be discussed in the next section. Consequently, it is difficult to accurately determine the structural effects of the αD26Y/βL98W TCR on
recognition of MART-1 and the MART-1 homologs since the affinity of this TCR most likely affected T cell function. Overall, the combination of the αD26Y TCR mutation and the βL98W TCR mutation in the αD26Y/βL98W TCR did not enhance MART-1 specificity.

Our positive and negative design strategy hypothesized that introduction of a TCR mutation that weakened TCR binding with the MHC would reduce off-target cross-reactivity. As predicted, the addition of the αY50A mutation to the αD26Y/βL98W TCR reduced the percentages of MART-1 homolog CD4+ reactive αD26Y/αY50A/βL98W TCR expressing T cells compared to αD26Y/βL98W TCR expressing T cells (Figure 19). However, compared to the T cells expressing the WT TCR, the percentages of MART-1 9mer and 10mer CD4+ reactive αD26Y/αY50A/βL98W TCR expressing T cells was significantly reduced (Figure 19). These results indicated that even though MART-1 homolog recognition was comparable, MART-1 specific reactivity was reduced. However, these results were different with the introduction of a different MHC weakening TCR mutation, αY50V. T cells expressing the αD26Y/αY50V/βL98W TCR exhibited comparable percentages of MART-1 9mer and 10mer reactive CD4+ T cells compared to T cells expressing the WT TCR (Figure 19). Also, there were no significant changes in the percentages of MART-1 homolog reactive T cells, however, minor increases were observed. These results indicated the introduction of a valine mutation at position 50 in the TCR alpha chain could offset the effects of the αD26Y/βL98W TCR and restore MART-1 reactivity to similar levels observed with the WT TCR. These results also indicated the importance of eliminating contact with positions 154, 155, and 158 in the HLA-A2 with the alanine mutation, to result in reduced T cell reactivity.
compared to the WT TCR levels. Overall, these results demonstrated the combination of the MHC weakening TCR mutations with peptide binding TCR mutations could alter on-target and off-target antigen recognition.

The effects of these structure-guided TCR mutations are generally described in a very linear fashion, based on their impact on the MART-1 decamer/HLA-A2 complex. However, the TCR/pMHC interaction includes only a small subset of all the membrane-bound proteins on a T cell and the APC. Thus, this interaction is very dynamic and fluid complex. It is clear from our data that structural predictions based on this complex, do not always directly correlate with recognition of altered-peptide ligands and cross-reactive peptides. This is likely due to multiple factors involved in the TCR/pMHC interaction. For example, it has been shown that anchor residues can alter TCR binding and specificity [475-478]. Therefore, it is possible that a peptide with little core sequence homology but an optimal anchor residue could impact TCR binding and recognition differently than a peptide with more core sequence homology but a suboptimal anchor residue. Specifically, it has been demonstrated that different anchor modifications of the MART-1 9mer and 10mer peptides elicited different cytokine responses [479]. Also, as already mentioned above, multiple favorable and non-favorable interactions within the TCR/pMHC complex will affect binding and subsequent signaling. These interactions will be different with each TCR and each ligand. Overall, these results emphasized the idea that the net effect of individual interactions within the TCR/pMHC complex dictates binding and peptide recognition.
**Correlation to 3D Affinity**

The structure-guided DMF5 TCR mutations altered the binding affinity compared to the WT DMF5 TCR. The DMF5 TCR 3D affinity measurements generally correlated to the percentages of reactive CD4+ T cells against MART-1 and the MART-1 homologs. One exception is the comparable percentages of reactive T cells between the WT TCR and αD26Y/αY50V/βL98W TCRs despite the αD26Y/αY50V/βL98W harboring a much lower affinity. Albeit, this result coincided with our goal in that using this strategy was not necessarily to enhance TCR affinity, but rather to redistribute the TCR’s free binding energy. Moreover, these results begin to elucidate the limitations associated with using the 3D affinity measurement against the target antigen as a predictor for cross-reactivity against altered-peptide ligands. For example, 4.9% of CD4+ T cells expressing the αD26Y/αY50V/βL98W recognized the MART-1 homolog, HREEP5, whereas only 2.5% of T cells expressing the βL98W TCR recognized HREEP5. Their 3D affinities with the MART-1 9mer are 140 μM and 12 μM, respectively. Despite the decrease in 3D affinity against the MART-1 9mer, the structural interaction between the αD26Y/αY50V/βL98W TCR and the HREEP 5/HLA-A2 complex elicited a better T cell response than with the βL98W TCR. The 3D affinity measurement is based upon the net interaction between the TCR/pMHC. However, it is possible that strongly enhanced binding, or strong favorable interactions at certain TCR/pMHC residues can heavily influence a T cell response, despite the presence of unfavorable interactions at other TCR/pMHC residues. Specifically, the net interactions will influence 3D affinity, but interactions at different TCR/pMHC residues could differentially influence conformational changes within the TCR/CD3 complex and subsequent signaling and T
cell function. In summary, the 3D affinity measured with the targeted ligand can correlate to functional outcomes with the targeted ligand and with altered-peptide ligands, but as demonstrated here, that is not always the case.

The field generally considers high affinity TCRs are optimal for T cell function and therapeutic efficacy. However, more recently it has been demonstrated when TCR affinity is too high, reduced T cell function is observed [146, 480-483]. This was exhibited in T cells expressing the αD26Y/βL98W TCR, as high TCR 3D affinity does not always yield enhanced T cell function. The lack of rational correlation between 3D affinity and T cell function in T cells expressing the αD26Y/βL98W TCR can be attributed to its supraphysiological high affinity. It has been demonstrated when a TCR’s 3D affinity approaches 5 - 1 μM, a decline in TCR signaling, in expression of activating and co-stimulatory molecules, and in T cell function is observed [146, 480-483]. SHP-1 negatively regulates TCR signaling and has been shown to be upregulated in an affinity-dependent manner [419, 484]. The highest levels of SHP-1 have been observed in T cells with supraphysiological TCR affinities [419]. Therefore, it would be of interest to determine the levels of SHP-1 in T cells expressing the αD26Y/βL98W TCR in response to MART-1 and the MART-1 homologs compared to T cells expressing the other DMF5 TCRs. Additionally, prolonged half-lives of TCRs have also been associated with a decrease in T cell function [485-488]. Serial triggering is important for full T cell activation and can be limited by prolonged half-lives [147]. It has also been shown that dissociation rates better correlate to T cell potency than TCR affinity [489-491]. $K_{on}$ and $K_{off}$ rates of the αD26Y/βL98W TCR could help elucidate some of the kinetic factors attributing to the observed decline in on-target T cell function. Additionally, we might
expect stark enhancements in the percentages of MART-1 homolog reactive αD26Y/βL98W TCR expressing T cells compared to the αD26Y TCR if we used 3D affinity as a predictor. Lastly, the 3D affinity measured with the αD26Y/βL98W TCR and the MART-1 decamer/HLA-A2 was in the nM affinity range. Therefore, MART-1 homologs harboring a superior anchor residue or other favorable interactions could again, result in an attenuated T cell responses with a high affinity TCR. In conclusion, these results suggested the supraphysiological 3D affinity of the αD26Y/βL98W TCR attenuated its on-target, and possibly off-target T cell potency in αD26Y/βL98W TCR expressing T cells.

It is well appreciated that higher affinity TCRs can be more cross-reactive [179, 483]. High affinity TCRs can tolerate significant changes in the peptide and still induce T cell activation, whereas these peptide changes with a lower affinity TCR could result in the loss of T cell activation [148]. This was exemplified in the MAGE-A3/Titin story. The affinity enhanced MAGE-A3 TCR recognized MAGE-A3 and Titin very similarly despite differences in the peptide core sequences [298]. This was also exemplified in our hands, with the αD26Y/βL98W DMF5 TCR. The αD26Y/βL98W TCR modeled with the MART-1 9mer peptide, HSV-1 glycoprotein III peptide, and M. tuberculosis protein I peptide indicated no substantial changes to the TCR CDR loops and side chains in the three different models [350]. The HSV-1 glycoprotein III peptide differs from the MART-1 9mer peptide in three residues and the M. tuberculosis protein I peptide differs from the MART-1 9mer peptide in seven residues, even in the peptide core. This demonstrated how the high affinity TCR accommodates structural differences in the peptide. In summary, 3D affinity can generally predict overarching patterns of antigen specificity.
and cross-reactivity in CD4+ T cells, however, this is not always the case, and is TCR and ligand-dependent.

**Contribution of CD4 and CD8**

Another factor that affects antigen specificity and cross-reactivity is the CD4 and CD8 co-receptors. Our data indicated the presence of the CD8 co-receptor increased the percentage of cross-reactive T cells compared to CD4+ T cells (Figures 19-20). This is not surprising due to the functional role of CD8 [92], and previous studies describing CD8’s role in cross-reactivity [148, 368]. CD8 stabilizes the TCR/pMHC interaction and furthermore, has the ability to influence $K_{on}$ and $K_{off}$ rates [421, 422, 492-494]. Specifically, CD8 has been shown to strengthen binding of TCR/pMHC by 3- to 4-fold [422, 494, 495]. Therefore, stabilization of low affinity interactions via CD8, with the aid in Lck recruitment, could induce T cell activation and T cell responses. This was apparent in the enhanced percentages of MART-1 homolog reactive WT TCR expressing CD8+ T cells compared to the CD4+ T cells. However, the specific role of CD8 becomes more complex within different TCR and ligand interactions. For example, CD8’s role in both TCR/pMHC stabilization and Lck localization have been implicated important for T cell function in lower affinity interactions, whereas only the role in Lck localization was important for T cell function in higher affinity interactions [439, 496]. Furthermore, we did not observe notable differences in the percentages of MART-1 and MART-1 homolog reactive CD4+ and CD8+ T cells expressing the high affinity αD26Y/βL98W TCR. There are two plausible explanations for this result. First, CD8 stabilization of a high affinity interaction could prolong the dissociation rate and reduce serial triggering, resulting in reduced T cell function or partial activation. Secondly, a
high affinity TCR, 17 μM, previously demonstrated comparable T cell function with and without the CD8 co-receptor [439]. Thus, implicating in a high affinity interaction, CD8 does not affect T cell function. However, the 3D affinity measurement of the βL98W TCR with the MART-1 9mer was 12 μM and exhibited notable differences between the percentages of MART-1 9mer CD4+ (25.9%) and CD8+ (41.9%) reactive T cells. This suggests the specific role of CD8 is TCR and ligand dependent.

The effect of the MART-1 targeting TCR mutations, αD26Y and βL98W, on MART-1 recognition was also different in CD4+ and CD8+ T cells. Although not significant, T cells expressing the αD26Y TCR and βL98W TCR did enhance the percentages of MART-1 reactive T cells in CD4+ T cells compared to T cells expressing the WT TCR but not in CD8+ T cells. These results indicated that the effect of the binding enhancement on MART-1 due to these TCR mutations was potentially overshadowed in the presence of the CD8 co-receptor. These results indicated that this structure-guided mutation strategy could have different implications in CD4+ and CD8+ T cells. For example, the positive and negative design worked in CD8+ T cells for reducing cross-reactivity, as T cells expressing the αD26Y/αY50A/βL98W exhibited a reduction in the percentage of MART-1 homolog reactive T cells compared to T cells expressing the WT TCR. However, WT TCR expressing T cells were barely reactive against the MART-1 homologs in the CD4+ T cells. Furthermore, MART-1 potency was significantly reduced in αD26Y/αY50A/βL98W TCR expressing CD4+ T cells compared to with the WT TCR. Therefore, the positive and negative design could be more important for reducing cross-reactivity in CD8+ T cells. Alternatively, although not significant, CD4+ T cells expressing the βL98W TCR enhanced the percentage of MART-1 reactive T cells
compared to the WT TCR, with some minor increases in the percentages MART-1 homolog reactive T cells. This enhanced percentage of MART-1 reactive T cells was not observed in the CD8+ T cells between the two TCRs. These results suggest that in CD4+ T cells, positive design could be more important in enhancing on-target potency, but most importantly, if the introduced TCR mutations do not, or very minimally, affect off-target recognition. In summary, CD8 augments cross-reactivity and masks the potential effect of peptide targeting mutations in the enhancement of antigen specific T cell potency. Therefore, the positive and negative design approach could have different therapeutic implications in CD4+ and CD8+ T cells.

**Cytotoxicity vs. Cytokine Expression**

It was notable that cytotoxicity against the HLA-A2+ melanoma tumor, MEL 624, did not correlate with 3D TCR affinity or polyfunctionality. Using 3D TCR affinity as a predictor, we would have expected T cells expressing the αD26Y/αY50A/βL98W or αD26Y/αY50V/βL98W TCRs to elicit reduced targeted melanoma killing compared to T cells expressing the WT TCR. However, T cells expressing the αD26Y/αY50A/βL98W TCR or the αD26Y/αY50V/βL98W TCR exhibited comparable or a trend towards better killing, respectively, compared to T cells expressing the WT TCR. If cytotoxicity directly correlated with CD107A and cytokine expression, we also would not have expected this based upon the total percentages of functional (CD107A and or/cytokine expression) T cells elicited against the MART-1 9mer.

There is evidence in the literature that explains how TCR affinity and T cell cytokine production do not always correlate with cytotoxicity. Initially, it had been shown that it is possible for just one TCR/pMHC interaction to induce a cytolytic T cell
response [151, 497]. Subsequent studies indicated three TCR/pMHC interactions elicited targeted cell death [498]. This is thought to be important for efficient immunity because a small number of antigenic pMHC complexes can be expressed on a target cell. Thus, cytotoxicity was determined to be the most antigen sensitive effector T cell response [499]. It was also demonstrated that using TCRs that varied in affinity, by up to 9-fold, resulted in similar killing. Furthermore, the induced cytotoxicity was elicited in transient immune synapse formation and reduced calcium flux [498]. This is suggests that T cell targeted killing is less dependent upon affinity and can occur in the absence of full T cell activation [500]. These findings coincide with our findings. Even though T cells expressing the two highest 3D affinity TCRs (αD26Y and αD26Y/βL98W) did significantly elicit a higher percentage of tumor killing compared to T cells expressing the WT TCR, cytotoxicity was comparable with the other higher and the lower affinity TCRs. Overall, these results indicate that 3D affinity does not always correlate to cytotoxicity.

Our cytotoxicity results also did not directly correlate with the observed polyfunctional T cell responses. It has been shown that cytokine production is more dependent upon kinetic factors. Cytokine production requires sustained signaling and maintenance at the immunological synapse for maximum effect [501, 502]. Altered-peptide ligands can also act as agonists, partial agonists, or antagonists to elicit different T cell responses. For example, lower affinity interactions have been shown to elicit cytokine production, but not T cell proliferation [150, 503]. Partial agonistic peptides have been demonstrated to elicit cytotoxicity and/or release of IFN-γ and TNF-α, but not IL-2 [328]. Partial agonists and antagonists tend to have shorter half-lives and
thus, potentially dissociate from the TCR before full T cell activation is achieved [79, 487, 489, 504]. It has been demonstrated that half-lives between 8 and 12 seconds allow all six tyrosines on the ITAMS to be phosphorylated, whereas half-lives between 3 and 8 seconds only allow enough time for a subset of the ITAMS to be phosphorylated, resulting in partial T cell activation [505]. In summary, the release of lytic granules and the expression of cytokines are governed by different mechanisms in T cells [506].

Based on the percentage of MART-1 reactive T cells, our results suggest that T cells expressing the αD26Y/αY50A/βL98W TCR could result in partial activation. Coinciding with our results, cytokine production is more correlated to 3D affinity and kinetic TCR/pMHC parameters, where cytotoxicity is less dependent upon TCR affinity.

Lastly, our results indicated that the percentages of CD107A+ T cells in response to the MART-1 9mer (Figures 35-36) generally correlated with cytotoxicity. The one exception to this correlation was observed with T cells expressing the αD26Y/βL98W TCR. Specifically, compared to T cells expressing the WT TCR, αD26Y/βL98W TCR expressing T cells exhibited a statistically significant increase in MEL 624 killing (Figure 17), but a statistically significant decrease in the percentage of CD107A+ CD8+ MART-1 9mer reactive T cells and a comparable percentage of CD107A+ CD4+ MART-1 9mer reactive T cells (Figure 36). It was notable that the drastic lack in correlation was observed with the TCR with the supraphysiological affinity. These results suggested at this level of affinity, CD107A expression is not correlative to lysis and/or CD107A expression is antigen density dependent. Following the results obtained from the off-target tumor panel, it is plausible to hypothesize that T cells expressing the modified DMF5 TCRs were exhibiting lysis in response antigens other than MART-1 on MEL 624
cells. However, the patterns in recognition of MEL 624 in terms of IFN-γ production, generally correlated with patterns observed in the percentages of MART-1 9mer reactive CD8+ T cells in Figure 20. It is possible the DMF5 TCRs recognized other antigens on MEL 624, however, these results implicated that was not the likely or sole justification. We would have to knockout MART-1 in MEL 624 tumor cells to determine the level of recognition due to other antigens. Overall, lysis broadly correlated to expression of CD107A except in T cells harboring a TCR with supraphysiological affinity.

**Polyfunction and Donor Variability**

Another factor that affects T cell responses is the donor in which T cells were derived. Our results indicated that T cells expressing the same TCR elicited different polyfunctional T cells responses in different donors even when seeing the same ligand. This was not surprising, as there are several factors that contribute to the T cell response. The structure of the TCR/pMHC interaction affects numerous biophysical parameters (i.e. affinity, on-rate, off-rate, t1/2, serial triggering, force) and then the combination with numerous cellular parameters (CD4/CD8, co-stimulatory molecules, ligand density, receptor density, activation status, exhaustion status, signaling molecules, TCR mispairing) subsequently dictates a polyfunctional response by a T cell. The T cell response is initiated by the TCR/pMHC interaction. Thus, the first variable here is the structure of the TCR/pMHC tri-molecular complex. This interaction affects numerous biophysical/kinetic parameters such as affinity, on-rate, off-rate, t1/2, serial triggering, and force. How these parameters can affect T cell function has been described in previous sections. The ability of a mutated TCR to alter T cell responses
compared to the WT TCR has also been previously demonstrated [507]. However, the kinetic factors are not the sole contributor to T cell function. There are numerous cellular factors that affect T cell function as well (CD4/CD8, co-stimulatory molecules, ligand density, receptor density, activation status, exhaustion status, signaling molecules, TCR mispairing).

First, a heterogeneous T cell population was used for generating TCR transduced T cells. This included T cells of various functional subsets and numerous specificities [508]. This can affect the T cell response for a number of reasons. For example, T cells exhibiting a stem cell memory or central memory phenotype demonstrated a more potent antigen response than effector memory T cells and long-term immune responses [509, 510]. Efficacy of CAR T cell therapy has also been associated with the proportion of naïve vs. effector T cells in the final product [511]. Furthermore, memory T cells have been shown to be stimulated by significantly lower peptide concentrations than naïve T cells, and have demonstrated faster and more efficient T cell function than naïve T cells [130, 512]. Therefore, the proportions of these T cell subtypes in different donors could affect their polyfunctional T cell response. An analysis of PBL from healthy patients demonstrated the percentage of naïve and memory CD8+ T cells varied widely among patients [513]. Different subsets of T cells express varying levels of activation and co-stimulatory molecules [514]. Additionally, one study demonstrated the donor variation in immune cell subpopulations as well as signaling responses in 60 healthy donors. Specifically, this study showed that IL-2 responses in naïve CD4+ T cells varied widely among the healthy donors [515]. Lastly, one study of 123 healthy donors indicated donors over the age of 40 exhibited lower
activation markers on CD8^+ T cells and lower naïve and central memory CD8^+ T cells compared to donors under the age of 40 [516]. These results illustrate the wide potential for donor variability associated with a heterogeneous PBL population.

Donor variability has been exemplified in numerous clinical trials both in terms of clinical responses and adverse events. When the WT DMF5 TCR was used clinically, objective cancer regressions were seen in 30% of the patients. Furthermore, on-target, off-tumor adverse events of varying degrees were seen in 16/20 patients [288]. If the T cells all were transduced the same way the same TCR, why didn’t every patient exhibit a clinical response or toxicities? Or why were they exhibited at different levels? In the MAGE-A3 clinical trial that resulted in lethal neurological toxicities, two out of nine patients died. However, five patients did not exhibit any neurological toxicities. Two patients exhibited neurological toxicities that were not lethal. Additionally, one patient exhibited a complete response and four patients exhibited a partial response [5]. In a clinical trial using NY-ESO-1 specific TCR engineered T cells, 16 out of 20 treated patients exhibited clinical responses [302]. These are a few examples of results that demonstrated the variety of T cell responses that can occur in different donors despite expressing the same introduced TCR. In summary, the structure of the TCR/pMHC impacts numerous kinetic factors that influences T cell function. However, numerous cellular factors further dictate the T cell response. Overall, donor variability and experimental variability occur for numerous reasons, therefore, it is not feasible to predict or associate a polyfunctional phenotype with a specific structural TCR/pMHC interaction.
Importance of MHC Weakening Site

One of the unique aspects of this project is combining TCR mutations that target the peptide, with TCR mutations that weaken binding with the MHC. Although peptide targeting TCR mutations would be ligand-dependent and thus TCR specific, the MHC targeting TCR mutations could be translatable to other TCRs. Our results indicated the translatability of the CDR2 region mutation in the TCR alpha chain that weaken binding with the MHC. More specifically, TCR mutation weakened or eliminated binding with positions 154, 155, and 158 in the HLA-A2 α2 helix with a valine or alanine mutation, respectively [350]. Residues at position 50 the CDR2 TCR alpha chain make one of the most frequent contacts between TCRs and MHC class I [37, 398]. These single TCR mutations in the DMF5 TCR eliminated all detectable reactivity against MART-1 loaded targets, further supporting the importance of this residue in pMHC binding and their evolutionarily selection. In the HCV 1406 TCR, alanine mutation at the same TCR contact site eliminated all detectable reactivity against the WT NS3 peptide loaded targets. However, the valine mutation eliminated all detectable reactivity against some of the mutant NS3 epitope loaded targets and reduced reactivity against other mutant NS3 epitopes and the WT NS3 peptide loaded targets compared to the WT TCR. These results indicated that these mutations impact HLA-A2 restricted TCRs differently, but yield a similar trend in eliminating or reducing off-target peptide recognition. Therefore, this conserved TCR alpha chain residue could be mutated in high affinity and/or potentially cross-reactive TCRs to reduce HLA-A2 restricted cross-reactivity. More importantly, this strategy could again be implemented in the positive/negative design strategy with other TCRs. This contact site has been broadly implicated in MHC class I
interactions, and not just limited to specifically HLA-A2. Furthermore, the alanine substitution had more of an impact than the valine substitution. Therefore, it is feasible to hypothesize that a smaller amino acid substitution, such as a glycine, would have even more of an impact than the alanine substitution. It would be interesting to determine how mutation in the TCR at this contact site in the MAGE-A3 affinity enhanced TCR would affect cross-reactivity with Titin, even though this TCR is HLA-A1 restricted. Furthermore, similar residues have been identified for MHC class II binding, indicating this strategy could be implemented in TCRs in CD4+ T cells as well [37]. Even though peptide targeting mutations would be TCR and ligand specific, this TCR alpha chain residue is seemingly broadly applicable to MHC class I restricted TCRs. In summary, this CDR2 TCR alpha chain residue could be translatable to other TCRs as a strategy to reduce potential cross-reactivity. Importantly, the unique role of the negative design in this novel positive and negative design strategy can be implemented in other TCRs.

**How DMF5 Mutations Affect Recognition of Processed Antigens**

**Correlation to MART-1 Homologs**

It is important to also observe recognition of processed antigens because exogenous peptide loaded T2 cells are not entirely biologically relevant. Furthermore, many peptide reactive T cells do not recognize processed antigen. The patterns of T cell reactivity against the MART-1 homologs by T cells expressing each of the different DMF5 TCRs did not directly correlate with their cross-reactivity against off-target tumors. These results exemplify the differences in conclusions that can be obtained when observing cross-reactivity via two distinct methods.
It is evident that utilizing peptides derived from a sequence homology search with the target ligand are not always adequate in assessing inclusive cross-reactivity. T cell cross-reactivity has been described based upon peptides with sequence homology, peptides with very minimal sequence homology but also, with unrelated peptides [517]. It is known that TCRs can recognize divergent peptides that would not be found in homology searches [350, 518]. Estimations suggest that one TCR can recognize one million different peptides [519]. This is possible due to their inherent plasticity in the CDR loops. TCR protein adaptability has been described and is critical for recognition of diverse peptides [474, 520]. Therefore, it is thought that cross-reactivity is not due to recognizing numerous unrelated peptides, but rather the tolerance of peptide alterations, and conservative alterations at residues that contact the TCR CDR loops [521]. These principles highlight the pitfalls associated with focusing solely on sequence homology as a predictor for cross-reactivity. An example of this was exemplified in our data. T cells expressing the αD26Y/αY50A/βL98W TCR did reduce cross-reactivity against the MART-1 homologs compared to T cells expressing the WT TCR. However, recognition of the off-target tumors was either comparable or enhanced with T cells expressing the αD26Y/αY50A/βL98W TCR compared to T cells expressing the WT TCR. In conclusion, we observed two very different results dependent upon the method in which we examined cross-reactivity. These results highlight the importance of addressing cross-reactivity in an inclusive manner.

Multiple studies indicated molecular mimicry could occur with structural and chemical similarities and not sequence similarities, thus implicating the importance of the antigenic peptide surface [517, 522-525]. Moreover, TCR recognition can rely on
sequence similarities in few regions of the peptide while tolerating vast diversity elsewhere in the peptide [369, 521, 526]. Specifically, it has been shown that just one conserved residue in the peptide can still result in partial T cell activation [527]. If T cells are estimated to recognize up to one million peptides, focusing on epitopes that have homology with only one of those potential million recognized peptides, is actually a very incomplete pool of potentially recognized targets. Based on what is known about TCR cross-reactivity and recognition of sequence dissimilar epitopes, it is understandable how focusing on MART-1 homologs, selected upon sequence homology, did not correlate to cross-reactivity against an abundant number of antigens on the off-target tumor cell lines. Furthermore, the TCRs harboring the structure-guided mutations did not always directly correlate to recognition of MART-1 homologs according to their sequence and the proposed structural effect of the specific TCR mutation. In summary, this inconsistency can be explained by the idea of TCRs recognizing structural mimicry as well as sequence mimicry.

The DMF5 TCR was identified based upon its recognition of MART-1. Accordingly, peptides in the MART-1 homolog peptide panel were selected based upon sequence homology with MART-1. However, the DMF5 TCR actually commonly recognizes two distinct classes of epitopes. One class is the hydrophobic motif, GIG, in the peptide core, and the second class is a central core consisting of charged amino acids [404, 423]. The DMF5 TCR tolerates such different antigenic surfaces by rotating its side chains to optimize electrostatic interactions [404]. Other TCR adaptability mechanism can involve CDR loop conformational changes or TCR repositioning [528-530]. The lack of conformational changes or repositioning indicates the DMF5 TCR
requires minimal alterations to accommodate divergent changes in the peptide core. MART-1 and the MART-1 homologs used in these studies fall into the peptide class with a hydrophobic motif in the peptide core. It would have been of interest to also investigate how each of the DMF5 TCRs recognize peptides in the second peptide class, consisting of central cores dominated by charged amino acids. The structure-guided mutations were designed based on the hydrophobic MART-1 peptide with the GIG peptide core, and thus, we are unsure of how these mutations in the DMF5 TCR would affect recognition of this other class of peptides. Therefore, antigens in this class of peptides, with a central charged core, could be expressed on the off-target tumors and were potentially recognized targets, however, we are unaware of how the different DMF5 TCRs recognize them.

Notably, our data generated by the combinatorial peptide libraries indicated enhanced recognition of peptides containing an aspartic acid at position 5 by cells expressing the αD26Y/αY50A/βL98W TCR compared to the WT TCR. Furthermore, this pool is recognized starkly more than all the other pools by cells expressing the αD26Y/αY50A/βL98W TCR. These results indicated that the αD26Y/αY50A/βL98W TCR might easily conform to or even prefer peptides with this antigenic surface. It is possible that the αD26Y mutation in this TCR removes an unfavorable interaction between the two negative charges that is present with the WT TCR. Furthermore, the removal of this repulsion is strong enough to not be offset by the αY50A mutation. T cell potency against this pool is enhanced with cells expressing the αD26Y/βL98W TCR compared to cells expressing the WT TCR. However, the addition of the αY50A mutation to the αD26Y/βL98W TCR was not enough to restore T cell reactivity to the
level observed with the WT TCR. Consequently, it is possible that peptides that conform to structures like the peptides in this specific pool, with an aspartic acid at the core of the peptide, could be presented on the off-target tumor lines. This would explain why T cells expressing the αD26Y/αY50A/βL98W TCR were more cross-reactive against some of the off-target tumors than T cells expressing the WT TCR.

It was demonstrated that the WT DMF5 TCR does exhibit a conserved interaction upon binding a peptide with the hydrophobic GIG core and a peptide with the charged core. This conserved interaction consisted of the CDR1 loop in the TCR alpha chain and the peptide backbone near the N-terminus [404]. This is interesting because the αD26Y mutation was made in the CDR1 loop targeting the N-terminal region of the peptide. Furthermore, TCRs containing this mutation were the most cross-reactive against the off-target tumors amongst the mutated DMF5 TCRs, with the βL98W TCR, the only mutated TCR lacking the αD26Y mutation, being the least cross-reactive.

These results suggest an important role of this mutation. If the interaction between the CDR1 TCR alpha chain loop and the N-terminal region of the peptide is conserved despite divergent peptides in the HLA-A2 complex, then the αD26Y mutation would be in the same conformation in every interface, and thus, could non-specifically enhance binding to any peptide. Our results suggested the structural role of the αD26Y mutation, in a conserved interaction, could be contributing the unpredicted off-target recognition of the off-target tumor lines.

It is possible for mutated TCRs to exhibit one pattern of cross-reactivity against certain classes of peptides while displaying a different pattern of cross-reactivity against a different class of peptides. This is most likely the reason why our results using the
MART-1 homologs did not directly align with reactivity against processed antigens on off-target tumor lines. Our results and other studies demonstrated valuable information that can be revealed using combinatorial peptide libraries. This will be discussed in a later section. In summary, when assessing TCR cross-reactivity, the sole use of peptides with sequence homology is not always a completely sufficient method.

3D Affinity vs. 2D Affinity

Our results indicated that 3D measurements better correlated with cross-reactivity against the MART-1 homologs, or with sequence homology, whereas 2D measurements better correlated with cross-reactivity against off-target tumor cells. There are numerous reasons why the 2D affinity measurements might better correlate to the more biologically relevant system. 2D measurements take into account the geometric and physical constraints involved in membrane-bound interactions, where 3D measurements do not [156, 166]. Thus, there are many intrinsic T cell factors such as membrane organization and orientation, membrane anchor, cytoskeleton regulation, and TCR structure in the CD3 complex that are involved in 2D measurements [380, 531-534]. Additionally, proteins in 3D SPR are in solution, whereas in the 2D micropipette frequency assay, the pMHC and TCR are directly, and head on, brought into contact. This affects how the TCR and pMHC come in contact with each other and thus how they bind together. Our results begin to illuminate the caveat with predicting T cell function and cross-reactivity based on 3D affinity measurements [168].

3D affinity measures the in vitro molecular binding properties, while 2D affinity measures physiological in situ kinetics [534]. Thus, our data highlight the differences between binding specificity and functional specificity in regards to 3D affinity and 2D
Specifically, 3D affinity measurements better correlated with recognition of MART-1 and the MART-1 homologs. For example, compared to the WT TCR, T cells expressing the αD26Y/αY50A/βL98W TCR reduced T cell potency against MART-1 and the MART-1 homologs, aligning with a reduction in this TCR’s 3D affinity. However, when observing recognition of off-target tumors, the overall functional specificity of T cells expressing the αD26Y/αY50A/βL98W TCR better correlated to its 2D affinity, in relation to the WT TCR. Figure 45 depicts the rankings of cross-reactivity with the different DMF5 TCRs in regards to the MART-1 homologs and the off-target tumor panel and their correlation with 3D and 2D affinity. The ranking of MART-1 homolog cross-reactivity in Figure 45A is based up the results calculated in Table 7. It is evident that reactivity against the MART-1 homologs followed the pattern of enhanced 3D affinity, enhanced cross-reactivity. Exceptions are the αD26Y/βL98W TCR, due to supraphysiological high affinity, and the αD26Y/αY50V/βL98W TCR being comparable to the WT TCR in terms of cross-reactivity but not 3D affinity. The ranking of off-target tumor cross-reactivity in Figure 45B is based up the results calculated in Figure 41. DMF5 TCR ranking in regards to the cross-reactivity against the off-target tumors lines is different than with the MART-1 homologs, but directly aligns with the measured 2D affinity. As mentioned previously, 2D affinity measurements have been better correlated with T cell function. An example of this has also been demonstrated in the clinic, where the 2D affinity of TCR transduced T cells correlated to clinical outcomes in a small sample set of patients [290]. Specifically in three patients, patient one exhibited no clinical response to treatment and their TCR transduced T cells exhibited the lowest 2D affinity measurement against the targeted ligand [290].
Furthermore, the other two patients exhibited clinical and biological responses and their TCR transduced T cells exhibited a statistically significant increase in their 2D affinity measurements [290]. These results indicated that donor variability is a factor in 2D affinity and can have implications in clinical outcomes. Overall, observing general reactivity against only one peptide, specifically with sequence homology to the targeted peptide, better correlated to 3D affinity. Conversely, observing reactivity in the presence of numerous peptides better correlated to 2D affinity and can be associated with functional specificity.

**Potential Tumor Targets**

Based on our data, and what we know about T cell cross-reactivity, we believe the mutated DMF5 TCRs were capable of recognizing multiple different peptides that fit...
an antigenic structure that is tolerated by the TCRs. First, we can eliminate TCR chain mispairing as the sole cause because Jurkat 76 cells expressing the WT and modified TCRs still recognized some of the tumors (data not shown – experiments completed by our collaborators in the Baker lab, results obtained through personal communication). Jurkat 76 cells lack an endogenous TCR and thus, any expressed TCR is the properly paired introduced TCR. Although TCR chain mispairing is always a potential cause of autoimmunity, these results demonstrated that cross-reactivity was not exclusively due to mispairing.

In the combinatorial peptide library data, the abundant recognition of nearly all the pools by the αD26Y/βL98W TCR supports the theory of plausible recognition of multiple peptides. Additionally, our results from the alanine scan also provided insight into the potential number of targets the mutated DMF5 TCRs could recognize (Figure 16). As mentioned previously, Titin was identified as the cross-reactive peptide with the affinity enhanced MAGE-A3 TCR. This was identified by fixing residues important for recognition based on an alanine scan, and searching for homologous epitopes in proteins [299]. When we performed a similar search based upon our alanine scan data, the results are far more extensive. For instance, with the αD26Y TCR, positions 4 and 6 in the MART-1 9mer peptide appeared important for recognition of the MART-1 9mer (Figure 16). When we fixed position 4 and position 6 and used the ScanProsite tool to search the UniProtKB/Swiss database for XXXIXIXXX motifs in proteins in Homo sapiens (including splice variants), 6,391 matches are found. When we performed the same search for the MAGE-A3 motif based upon the authors alanine scan results (EXDPIXXXY), only 17 matches were found in Homo sapiens (including splice variants).
Albeit, this does not mean all 6,391 epitopes are presented on HLA-A2. Recent advancements in modeling, bioinformatics, and epitope-mapping have been made in order to better predict epitope presentation by MHC class I and MHC class II [535-542]. Nonetheless, this drastic difference illuminates the vast potential of targets that these modified DMF5 TCRs are recognizing. Furthermore, this does not even begin to address the potential recognized epitopes that do not follow MART-1 sequence homology. In summary, our results indicated mutations in the DMF5 TCR allowed the TCRs to be more permissive in their recognition and allowed for non-specific recognition of multiple different peptides.

There are a few methods that could be utilized to determine the recognized targets on the off-target tumor cells lines. One potential approach to determine the recognized targets would be acid eluting off the peptides from the HLA-A2 complexes [543-546]. Following elution, peptide extracts would be fractionated by reverse phased high-performance liquid chromatography (HPLC). Cytotoxicity assays would be performed with T2 cells loaded with peptide fractions and effector T cells. Further fractionation of reactive peaks would narrow down the number of potential peptides. Lastly, mass spectrometry would determine the sequence of peptides, and all potential peptides would be made and put in final functional assays. This approach has been utilized to identify an antigenic peptide (YXEPGPVTA) for melanoma specific T cell clones [546]. A second approach is using combinatorial peptide libraries in a positional scanning format [547-550]. A scoring matrix is generated based upon lysis of the specific amino acids at each residue. This matrix is then implemented with protein databases to predict stimulatory scores for epitopes present in proteins. This approach
was utilized to predict off-target reactivity of the 7B5 TCR (HLA-A2 restricted, recognizes the HA-2 peptide), and even identified a cross-reactive peptide. Notably, this study demonstrated that results from their alanine scan assay were insufficient for identifying this cross-reactive peptide [442]. However, these approaches might be impractical for identifying the recognized peptides on the off-target tumor cells in this study. We have already partially completed the combinatorial peptide library approach, and results indicated recognition of nearly all the pools by the WT and αD26Y/βL98W TCR, suggesting the extensive number of potential targets with a wide variety of potential sequences (Figure 44). Nonetheless, when used clinically, the DMF5 WT TCR did not cause adverse events due to off-target autoimmunity [332]. These results indicated that despite observation of off-target cross-reactivity in vitro, it was not observed in a human. Therefore, there could be a “cut off” point or range in which autoimmunity might occur in vivo. Based on what we know about donor variability, this cut off is not likely to be highly definitive. Furthermore, it is plausible that the threshold for causing off-target autoimmunity in vivo is TCR and ligand dependent. Albeit, the peptide elution or combinatorial peptide library approaches could be more practical for identifying the off-target peptides for the αD26Y/αY50A/βL98W TCR. Results from the combinatorial peptide library with this TCR, indicated far fewer peptide pools that elicited discernable reactivity, and thus, could be parsed apart more easily. Taken together, our results suggested the number of peptides recognized on the off-target tumor cells was potentially very extensive for the highly cross-reactive DMF5 TCRs (αD26Y TCR, αD26Y/βL98W TCR, and αD26Y/αY50V/βL98W TCR) but limited for the αD26Y/αY50A/βL98W TCR.
Implications for Future Use of Structure-Guided Design Strategy

The results from our studies have critical implications for future utilization of this structure-guided design strategy for manipulating TCR specificity. Some of our findings were expected. However, our results do not undervalue the structure-guided design strategy and its implications for future use. Importantly, our results provided critical insight for the field in terms of modifying TCRs and addressing cross-reactivity.

We initially thought the DMF5 TCR/MART-1 peptide model would be useful to study the cross-reactive properties of TCRs. This was based upon the reports of high frequencies of MART-1 reactive T cells in the blood of cancer patients and healthy donors [323, 327, 551], and the evidence that MART-1 “like” epitopes recurrently appear among self and non-self-proteins [327]. However, the inherent nature of this TCR and its ligand, could have exacerbated some of the findings compared to if a different TCR and ligand were used. For example, MART-1 9mer is extremely hydrophobic, being all hydrophobic except for the neutral threonine at position 8. Since immunogenicity and hydrophobicity have been directly correlated [68], it makes sense the DMF5 TCR is cross-reactive. However, recognition of hydrophobic peptides is already preferred by TCRs, compared to more polar peptides, because there is less defined geometry for the CDR loops to match [72]. Herein, we made peptide enhancing mutations to a TCR that was already very permissive in terms of cross-reactivity of sequence homologous and non-sequence homologous epitopes. One of the main challenges associated with the MART-1 peptide for our approach was that there are no unique residues to target that could be considered relatively MART-1 “specific”. Therefore, the combination with negative, MHC weakening, TCR mutation did not offset
the positive, MART-1 targeting, TCR mutation enough to prevent cross-reactivity against all potential targets. That being said, this positive and negative design strategy could be implemented in other TCRs and perceivably yield different results. This concept of the MART-1 9mer being very hydrophobic and “featureless” relates to the commonly described features of a “vanilla” peptide or a “spicy” peptide [552]. Spicy peptides are described as having a prominent feature exposed to the TCR, whereas vanilla peptides are described as being featureless. Thus, others have hypothesized that TCRs that recognize a spicy peptide should be less cross-reactive because most other peptides would lack the defining peptide feature. Conversely, TCRs that target a vanilla peptide should be more cross-reactive because peptide features are more shared amongst other peptides [552]. Our studies with the DMF5 TCR and other studies with MART-1 reactive T cells exemplify this hypothesis [327, 350]. Consequently, in TCRs that recognize less hydrophobic peptides, there could be more distinct peptide residues to target for positive TCR mutation. Thus, introduction of a TCR mutation that enhances binding to that distinct peptide feature, could further enhance antigen specificity. Subsequent addition of a negative, MHC weakening, TCR mutation, could then reduce any remaining potential off-target cross-reactivity. Every TCR/pMHC ligand will be different, but these results outlined preliminary stipulations to bear in mind when mutating TCRs and altering their functional specificities. In summary, it is plausible that this structure-guided strategy would yield different results with another TCR and ligand.

Our results also highlight the importance, but difficulty, in the introduction of a peptide targeting TCR mutation that only impacts on binding with the peptide and not the MHC. Specifically, our results indicated that the αD26Y mutation was most likely
driving off-target cross-reactivity and the structural effect of this mutation was too impactful to be completely countered balanced by the negative mutation. This was demonstrated in the both the first and the second round of DMF5 TCR mutations, where every mutant DMF5 TCR containing the αD26Y TCR mutation enhanced recognition of the off-target tumors, compared to mutant DMF5 TCRs that did not contain the αD26Y TCR mutation. As mentioned previously, we cannot discern the impact of the MART-1 enhancement of this mutation versus the MHC enhancement, but it could be imperative for future mutations to avoid also enhancing binding with the MHC molecule. Unfortunately, this is easier said than done, as TCR contact with the MHC accounts for 75-80% of the TCR/pMHC interaction, and the peptide and MHC are closely packed together [71, 349]. Furthermore, the impact of binding enhancement with the MHC could be TCR and ligand dependent. Specifically, it is possible that the removal of the charge repulsion between the HLA-A2 and the DMF5 TCR with the αD26Y TCR mutation was important for the observed enhancement in cross-reactivity. Conversely, it is possible that the introduction of a favorable interaction between the TCR and MHC in a different model would have a minor effect on cross-reactivity. A future strategy for this DMF5 TCR would be remove the αD26Y mutation from the αD26Y/αY50A/βL98W TCR and replace it with possibly another CDR3 region mutation that targets the core of the peptide. Overall, the goal of the positive design strategy was to introduce mutations that only impact peptide binding. Furthermore, this can be a difficult task, and implications are likely to be TCR and ligand dependent.

It was very interesting that just one or two mutations in the DMF5 TCR (αD26Y or αD26Y/βL98W) frequently elicited recognition of the off-target HLA-A2* tumors in our
panel. We believe that in the proper system, these TCRs could potentially be therapeutic. This system would be an off the shelf reagent of TCR transduced allogeneic T cells for intra-tumoral injection in an HLA-A2\(^+\) tumor (E. Fleming-Trujillo, et. al. unpublished). First, the TCR transduced T cells have to be allogeneic because, activated TCR transduced HLA-A2\(^+\) T cells commit fratricide. Secondly, it would have to be an intra-tumoral injection because it could be detrimental to systemically inject allogenic T cells expressing a highly cross-reactive TCR. The TCR transduced allogeneic T cells could also be irradiated to limit any potential damage. Furthermore, intra-tumoral injection of irradiated TCR transduced allogeneic T cells has already been demonstrated as feasible and safe, with the potential to elicit regression of untreated tumors [331]. In summary, we believe the off-target cross-reactive properties of the \(\alpha D26Y\) and \(\alpha D26Y/\beta L98W\) TCRs could be widely therapeutic as an off the shelf reagent for treatment of HLA-A2\(^+\) tumors, if implemented in the proper and safe system.

The results of this dissertation highlight the need for the advancement in modeling and prediction tools for protein interactions, specifically in the area of cross-reactivity. We demonstrated that when examining potential cross-reactivity, sequence homology searches are not always adequate, and furthermore, our panel of tumors did not encompass every tissue. Addressing cross-reactivity in murine models is also not completely sufficient, due to lack of complete homology between the mouse and human. At this point, there is no better model than a human to fully examine potential cross-reactivity, and even then, results are not identical. Immunogenicity predictions tools such as the Immune Epitope Database, are a step in the right direction to better predict and characterize B cell and T cell epitopes involved in disease, autoimmunity,
allergy, and transplant [541]. In summary, the advancements in modifying TCRs and their antigen specificities should coexist with the advancements in modeling and prediction tools.

**In vivo Future Directions**

Our data demonstrated some of the challenges associated with using xenograft in vivo models for our studies. Our results suggested that human TCR transduced T cells had difficulty trafficking to the site of the tumor, most likely due to the immunodeficient nature of the NSG mouse model and the lack of human environment. Aside from what was examined in these studies, there are other methods of trying to enhance T cell persistence and trafficking to the tumor that could be explored in the future. For example, IL-7 is important for T cell survival and differentiation. IL-7 has been shown to enhance adoptive T cell therapy, and more specifically, the combination of IL-7 and IL-15 [460, 553-556]. Thus, it would be of interest to determine how IL-7 or how the combination of IL-7, IL-15, and IL-2 affect the persistence and anti-tumor activity of the introduced TCR transduced T cells. In our engraftment model, engrafted T cells did persist, but did not enhance the anti-tumor activity of the introduced TCR transduced T cells. It is plausible that engraftment of whole hematopoietic stem cells would better recapitulate a human immune system than engrafted PBMCs due to the multiple hematopoietic lineages. Therefore, they might better support to persistence of the introduced T cells [385, 557-561]. Lastly, another option would be to murinize the TCR constant regions of DMF5 TCRs and transduce murine T cells. These TCR transduced murine T cells could be used in immunocompetent mice for analysis of anti-tumor activity. In conclusion, different cytokine support, humanized NSG mice, or
switching mouse models to immunocompetent mice are a few examples of alternative strategies to enhance the \textit{in vivo} anti-tumor efficacy and analysis of TCR transduced T cells in these studies.

\textbf{Concluding Remarks}

In these studies we developed and implemented a novel structure-guided approach to enhance antigen specificity and reduce off-target cross-reactivity. We sought to determine how structure-guided mutations in the DMF5 TCR altered tumor lysis, polyfunctional T cell responses, on-target specificity, and recognition of processed antigens. When measuring polyfunctional T cell responses against a panel of MART-1 homologs, the structure guided approach appeared to be promising, with T cells expressing the αD26Y/αY50A/βL98W TCR exhibiting reduced percentages of MART-1 homolog reactive T cells compared to T cells expressing the WT TCR. However, when T cell responses were measured against processed antigens on a variety of different tissues, large off-target cross-reactivity was observed. Our results demonstrate that sequence homology is not the sole factor in cross-reactivity, and that 3D affinity against the “cognate” antigen is not always correlative to broad cross-reactivity. Furthermore, T cell responses vary due to biophysical properties/kinetics of the TCR/pMHC interaction and how structural changes in the interface affect these properties and consequent downstream T cell function is not feasibly predictable. Despite this, our structure-based designed strategy could be utilized in the future, as it is more meticulous and specific than random mutation through yeast or phage display. It important to understand how alterations in the TCR/pMHC interface can affect functional T cell phenotypes to maximize the efficacy and safety of TCRs to be used in gene modified T cells in
adoptive cell transfer. In conclusion, when altering TCRs for therapeutic use, safety should be of the utmost importance and herein, we emphasize the importance of rigorous preclinical testing of modified TCRs and the need for advancement in modeling/prediction tools for protein interactions.
APPENDIX A:
ADDITIONAL FIGURES
Figure 46. Impact of DMF5 Mutations in TCR Transduced CD4+ T cells on Polyfunctional Responses against MART-1 (Donor One). The data indicate reactivity against T2 cells loaded with the MART-1 9mer peptide in donor one, experiment two. “Cool plots” were generated to include any functional phenotype present over 0.1%. In the functional phenotypes listed, “+” indicates positive for the marker and “-” indicates negative for the marker. As described in Chapter Two, reactivity is defined after background subtraction and specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). The color depicted in the “cool plot” correlates to the scale on the right. An “X” indicates no T cells were positive (<0.1% after background subtraction) for given functional phenotype. The color white for a given functional phenotype indicates a percentage less than 0.2%.
Figure 47. Impact of DMF5 Mutations in TCR Transduced CD4+ T cells on Polyfunctional Responses against MART-1 (Donor Two). The data indicate reactivity against T2 cells loaded with the MART-1 9mer peptide in donor two, experiment two. “Cool plots” were generated to include any functional phenotype present over 0.1%. In the functional phenotypes listed, “+” indicates positive for the marker and “-” indicates negative for the marker. As described in Chapter Two, reactivity is defined after background subtraction and specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). The color depicted in the “cool plot” correlates to the scale on the right. An “X” indicates no T cells were positive (≤0.1% after background subtraction) for given functional phenotype. The color white for a given functional phenotype indicates a percentage less than 0.2%.
Figure 48. Impact of DMF5 Mutations in TCR Transduced CD4+ T cells on Polyfunctional Responses against MART-1 (Donor Three). The data indicate reactivity against T2 cells loaded with the MART-1 9mer peptide in donor three, experiment two. “Cool plots” were generated to include any functional phenotype present over 0.1%. In the functional phenotypes listed, “+” indicates positive for the marker and “-“ indicates negative for the marker. As described in Chapter Two, reactivity is defined after background subtraction and specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). The color depicted in the “cool plot” correlates to the scale on the right. An “X” indicates no T cells were positive (≤0.1% after background subtraction) for given functional phenotype. The color white for a given functional phenotype indicates a percentage less than 0.5%.
Figure 49. Impact of DMF5 Mutations in TCR Transduced CD8+ T cells on Polyfunctional Responses against MART-1 (Donor One). The data indicate reactivity against T2 cells loaded with the MART-1 9mer peptide in donor one, experiment two. “Cool plots” were generated to include any functional phenotype present over 0.1%. In the functional phenotypes listed, “+” indicates positive for the marker and “-” indicates negative for the marker. As described in Chapter Two, reactivity is defined after background subtraction and specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). The color depicted in the “cool plot” correlates to the scale on the right. An “X” indicates no T cells were positive (<0.1% after background subtraction) for given functional phenotype. The color white for a given functional phenotype indicates a percentage less than 0.5%. Five prominent phenotypes are highlighted with red boxes.
Figure 50. Impact of DMF5 Mutations in TCR Transduced CD8+ T cells on Polyfunctional Responses against MART-1 (Donor Two). The data indicate reactivity against T2 cells loaded with the MART-1 9mer peptide in donor two, experiment two. “Cool plots” were generated to include any functional phenotype present over 0.1%. In the functional phenotypes listed, “+” indicates the positive for marker and “-” indicates negative for the marker. As described in Chapter Two, reactivity is defined as background subtraction and specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). The color depicted in the “cool plot” correlates to the scale on the right. An “X” indicates no T cells were positive (≤0.1% after background subtraction) for given functional phenotype. The color white for a given functional phenotype indicates a percentage less than 0.5%. Five prominent phenotypes are highlighted with red boxes.
Figure 51. Impact of DMF5 Mutations in TCR Transduced CD8+ T cells on Polyfunctional Responses against MART-1 (Donor Three). The data indicate reactivity against T2 cells loaded with the MART-1 9mer peptide in donor three, experiment two. “Cool plots” were generated to include any functional phenotype present over 0.1%. In the functional phenotypes listed, “+” indicates positive for the marker and “-” indicates negative for the marker. As described in Chapter Two, reactivity is defined after background subtraction and specifically, any phenotype expressed by over 0.1% of TCR transduced T cells (subtraction of the background immunofluorescence observed with T2 cells loaded with an irrelevant, HCV, peptide). The color depicted in the “cool plot” correlates to the scale on the right. An “X” indicates no T cells were positive (≤0.1% after background subtraction) for given functional phenotype. The color white for a given functional phenotype indicates a percentage less than 0.5%. Five prominent phenotypes are highlighted with red boxes.
Figure 52. Impact of DMF5 Mutations on Cross-Reactivity Using Multi-Tissue Tumor Panel. Human T cells expressing either WT or a mutant DMF5 TCR were stimulated with tumor cell lines (see Table 2 – Chapter Two, Methods). IFN-γ release was measured by ELISA in triplicate wells. SAUJ and MEL 624-28 are HLA-A2+, all other tumors are HLA-A2. Two patterns of off-target tumor recognition were observed. (a) Highly cross-reactive TCRs compared to the WT TCR. (b) Modestly cross-reactive TCRs compared to the WT TCR. One representative experiment and donor is shown. Reactivity is defined as a T cell culture that secretes twice background, or twice the amount of IFN-γ produced as against MEL 624-28, and greater than 200 pg/mL. Donor 1, experiment 2.
Figure 53. Impact of DMF5 Mutations on Cross-Reactivity Using Multi-Tissue Tumor Panel. Human T cells expressing either WT or a mutant DMF5 TCR were stimulated with tumor cell lines (see Table 2 – Chapter Two, Methods). IFN-γ release was measured by ELISA in triplicate wells. SAUJ and MEL 624-28 are HLA-A2⁺, all other tumors are HLA-A2*. Two patterns of off-target tumor recognition were observed. (a) Highly cross-reactive TCRs compared to the WT TCR. (b) Modestly cross-reactive TCRs compared to the WT TCR. One representative experiment and donor is shown. Reactivity is defined as a T cell culture that secretes twice background, or twice the amount of IFN-γ produced as against MEL 624-28, and greater than 200 pg/mL. Donor 2, experiment 1.
Figure 54. Impact of DMF5 Mutations on Cross-Reactivity Using Multi-Tissue Tumor Panel. Human T cells expressing either WT or a mutant DMF5 TCR were stimulated with tumor cell lines (see Table 2 – Chapter Two, Methods). IFN-γ release was measured by ELISA in triplicate wells. SAUJ and MEL 624-28 are HLA-A2⁺, all other tumors are HLA-A2⁺. Two patterns of off-target tumor recognition were observed. (a) Highly cross-reactive TCRs compared to the WT TCR. (b) Modestly cross-reactive TCRs compared to the WT TCR. One representative experiment and donor is shown. Reactivity is defined as a T cell culture that secretes twice background, or twice the amount of IFN-γ produced as against MEL 624-28, and greater than 200 pg/mL. Donor 2, experiment 2.
Figure 55. Impact of DMF5 Mutations on Cross-Reactivity Using Multi-Tissue Tumor Panel. Human T cells expressing either WT or a mutant DMF5 TCR were stimulated with tumor cell lines (see Table 2 – Chapter Two, Methods). IFN-γ release was measured by ELISA in triplicate wells. SAUJ and MEL 624-28 are HLA-A2+, all other tumors are HLA-A2+. Two patterns of off-target tumor recognition were observed. (a) Highly cross-reactive TCRs compared to the WT TCR. (b) Modestly cross-reactive TCRs compared to the WT TCR. One representative experiment and donor is shown. Reactivity is defined as a T cell culture that secretes twice background, or twice the amount of IFN-γ produced as against MEL 624-28, and greater than 200 pg/mL. Donor 3, experiment 1.
Figure 56. Impact of DMF5 Mutations on Cross-Reactivity Using Multi-Tissue Tumor Panel. Human T cells expressing either WT or a mutant DMF5 TCR were stimulated with tumor cell lines (see Table 2 – Chapter Two, Methods). IFN-γ release was measured by ELISA in triplicate wells. SAUJ and MEL 624-28 are HLA-A2+, all other tumors are HLA-A2+. Two patterns of off-target tumor recognition were observed. (a) Highly cross-reactive TCRs compared to the WT TCR. (b) Modestly cross-reactive TCRs compared to the WT TCR. One representative experiment and donor is shown. Reactivity is defined as a T cell culture that secretes twice background, or twice the amount of IFN-γ produced as against MEL 624-28, and greater than 200 pg/mL. Donor 3, experiment 2.
Figure 57. Impact of DMF5 Mutations on Cross-Reactivity Using Multi-Tissue Tumor Panel. Human T cells expressing either WT or a mutant DMF5 TCR were stimulated with tumor cell lines (see Table 2 – Chapter Two, Methods). IFN-γ release was measured by ELISA in triplicate wells. SAUJ and MEL 624-28 are HLA-A2+, all other tumors are HLA-A2−. Two patterns of off-target tumor recognition were observed. (a) Highly cross-reactive TCRs compared to the WT TCR. (b) Modestly cross-reactive TCRs compared to the WT TCR. One representative experiment and donor is shown. Reactivity is defined as a T cell culture that secretes twice background, or twice the amount of IFN-γ produced as against MEL 624-28, and greater than 200 pg/mL. Donor 4, experiment 1.
Figure 58. Impact of DMF5 Mutations on Cross-Reactivity Using Multi-Tissue Tumor Panel. Human T cells expressing either WT or a mutant DMF5 TCR were stimulated with tumor cell lines (see Table 2 – Chapter Two, Methods). IFN-γ release was measured by ELISA in triplicate wells. SAUJ and MEL 624-28 are HLA-A2+, all other tumors are HLA-A2*+ Two patterns of off-target tumor recognition were observed. (a) Highly cross-reactive TCRs compared to the WT TCR. (b) Modestly cross-reactive TCRs compared to the WT TCR. One representative experiment and donor is shown. Reactivity is defined as a T cell culture that secretes twice background, or twice the amount of IFN-γ produced as against MEL 624-28, and greater than 200 pg/mL. Donor 4, experiment 2.
Figure 59. Impact of Second Round of DMF5 Mutations on Cross-Reactivity Using Multi-Tissue Tumor Panel. Human T cells expressing either WT or mutant DMF5 TCR were stimulated with tumor cell lines for 18 hours (see Table 2 – Chapter Two, Methods). IFN-γ release was measured by ELISA in triplicate wells. Reactivity is defined as a T cell culture that secretes twice background, or twice the amount of IFN-γ produced as against MEL 624-28, and greater than 200 pg/mL.
Figure 60. Engraftment of PBMCs in NSG A2+ Mice. (a) Schematic of experimental design is depicted. (b) Persistence of human CD3+ T cells in the blood of non-engrafted and engrafted mice on day 72 post engraftment of human PBMCs. Cells were immunofluorescently labeled with a human anti-CD3 mAb. Each group consisted of 3 mice. One representative mouse per group is shown.
Figure 61. Effect of Engraftment on Therapeutic Efficacy of TCR Transduced T cell Treatment. (a) Schematic of experimental design is depicted. Mice were engrafted with PBMC on day -7 via retro-orbital injection. Mice were tumor challenged on day 0 with MEL 624 tumor cells. Mice were treated on day 17 with untransduced, WT DMF5 TCR transduced, αD26Y TCR, or αD26Y/αY50A/βL98W TCR T cells via retro-orbital injection. (b) Tumor growth of MEL 624. N = 3 mice/group. Data represent the mean ± standard deviation.
Figure 62. CD34 Expression Prior and Post in Vivo Injection. (a) Human untransduced, WT TCR expressing, αD26Y TCR, and αD26Y/αY50A/βL98W TCR expressing T cells were labeled with an anti-CD34 mAb 12 days after REP and 1 day prior to in vivo injection. (b) Persistence of human CD3+CD34+ TCR transduced T cells in the blood of treated and untreated mice on day 13 post therapeutic TCR transduced T cell injection. Cells were immunofluorescently labeled with human anti-CD3 and anti-CD34 mAbs. Each group consisted of 3 mice. One representative mouse per group is shown.
Figure 63. Effect of Cytokine Support on Therapeutic Efficacy of TCR Transduced T cell Treatment. (a) Schematic of experimental design is depicted. Mice were tumor challenged on day 0 with MEL 624 tumor cells. On day 17, mice were treated with untransduced or WT DMF5 TCR transduced T cells via retro-orbital injection. Beginning on day 17, cytokine treatment groups of mice were treated with 2.5 μg rhIL-15 every 3 days or 60,000 IU rhIL-2 twice a day for four days, then every 3 days via intraperitoneal injection. (b) Tumor growth of MEL 624. N = 3 mice/group. Data represent the mean ± standard deviation.
Figure 64. CD34 Expression Of Injected T Cells 10 Days Post *In Vivo* Injection. Persistence of human CD3^+^CD34^+^ TCR transduced T cells in the blood of mice treated with untransduced or WT TCR transduced T cells, with or without IL-2 or IL-15 cytokine support on day 10 post therapy. Cells were immunofluorescently labeled with human anti-CD3 and anti-CD34 mAbs. Each group consisted of 3 mice. One representative mouse per group is shown.
Figure 6. TCR Transduced T cells in the Tumor on Day 16 Post Treatment. On day 16 post treatment, tumors were harvested and processed from one mouse per group. Cells were immunofluorescently labeled with human anti-CD3 and anti-CD34 mAbs.
Figure 66. TCR Transduced T cells In the Spleen on Day 16 Post Treatment. On day 16 post treatment, spleens were harvested and processed from one mouse per group. Cells were immunofluorescently labeled with human anti-CD3 and anti-CD34 mAbs.
Figure 67. Effect of Anti-PD-1 on Therapeutic Efficacy of TCR Transduced T cell Treatment. (a) Schematic of experimental design is depicted. Mice were tumor challenged on day 0 with MEL 624 tumor cells. On day 17, mice were treated with untransduced or WT DMF5 TCR transduced T cells via retro-orbital injection. Beginning on day 17, anti-PD-1 groups of mice were treated with 0.25 mg of anti-PD-1 every 3 days via intraperitoneal injection. (b) Tumor growth of MEL 624. N = 3 mice/group. Data represent the mean ± standard deviation.
Figure 68. Schematic Of In Vivo CTL Assay. On day 0, PBS or effector T cells were injected via retro-orbital route. On day 1, $6 \times 10^6$ MART-1 9mer HLA-A2+ PBMCs labeled with a high concentration of CFSE and $6 \times 10^6$ HLA-A2+ PBMCs labeled with a low concentration of CFSE were injected via retro-orbital route. On day 3, spleens were harvested and processed and analyzed via flow cytometry for CFSE cells.
Figure 69. Example of CFSE Cells in the Spleen of PBS and WT DMF5 TCR Transduced T cell Treated Mice. Splenocytes from each group were analyzed via flow cytometry. Splenocytes were first gated on HLA-A2\(^+\) cells to differentiate the target cells from any murine splenocytes. Cells were then gates on SSC and CFSE. Depicted above is an example of CFSE low and high cells in a PBS treated mouse and CFSE low and high cells in a WT DMF5 TCR transduced T cell treated mouse.
Figure 70. MART-1 Specific Lysis by WT DMF5 TCR Transduced T Cells. One day post PBS or WT DMF5 TCR transduced T cell treatment, mice were treated with $6 \times 10^6$ MART-1 pulsed [0.5 μM] and $6 \times 10^6$ [5 μM] CFSE labeled HLA-A2+ PBMCs. Two days after injection of CFSE labeled target cells, spleens were harvested and processed. Proportions of CFSE labeled cells in the spleen were analyzed via flow cytometry. Data represent the mean ± the standard error of the mean. N = 5 mice/group.
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