ENGINEERING HUMAN SINGLE-CHAIN T CELL RECEPTORS

BY

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DISSERTATION

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ABSTRACT

The αβ T cell receptor (TCR) is responsible for mediating T cell recognition of self and non-self tissues, through recognition between a complex of a peptide and a product of the major histocompatibility complex (pepMHC) on target cells. In the immune response to cancerous tissue, the immune repertoire of T cells is often insufficient to target pepMHC complexes associated with cancer cells, as these tumor antigens have often induced tolerance or the tumor microenvironment promotes immunosuppression of T cells. To improve the response to tumors, gene therapy with tumor specific T cell receptors provides an attractive approach to effectively arm patient's T cells for cancer cell destruction. An inherent difficulty, however, is generation of T cell receptors of sufficient affinity to redirect both $CD4^+$ and $CD8^+$ T cells. This thesis describes the development of engineering strategies for human single-chain T cell receptor variable fragments (scTv), with the goal of understanding the properties that allow scTv to be expressed and deployed in a therapeutic mode. Stable scTv receptors can be used to generate high-affinity TCRs specific for disease-associated pepMHC complexes and produced in soluble expression systems for subsequent biochemical and biophysical characterization. This work also develops scTv and scFv (antibody variable fragments) as fusion proteins, collectively called chimeric antigen receptors, for cell mediated therapies to redirect T cells to specific antigens

In chapter 2, two human $V\alpha2^+$ T cell receptors specific for human immunodeficiency virus and human T cell lymphotrophic virus derived pepMHC complexes were engineered for improved stability as scTv proteins, consisting of only the variable domains of the T cell receptor attached by a flexible linker. High-affinity, stabilized scTv proteins could be expressed as soluble proteins in *E. coli* and used for detection of low levels of HIV pepMHC antigens, suggesting that these receptors have potential diagnostic applications for the detection of HIV infected cells. Finally, the results suggest that other $V\alpha2^+$ TCRs with different specificities can be engineered for enhanced affinity by yeast display.

Chapter 3 describes the development of chimeric antigen receptors, that consist of scTv-fusion proteins, for T cell targeting of tumor antigens. scTv proteins

engineered for improved stability by yeast display were fused to the intracellular signaling domains of CD28,CD3ζ, and LCK and introduced into murine T cells. The high affinity scTv, called m33, that is specific for the pepMHC SIY/K^b was used to redirect T cells with similar antigen sensitivity to the full-length m33 TCR. An inherent problem with full-length TCR gene therapy is the generation of receptors of unknown specificity through mispairing between introduced and endogenous TCR, leading to graft versus host disease or autoimmunity. I show that the scTv-fusions avoided mispairing with endogenous αβ TCRs and allowed for endogenous TCR surface expression at high levels. A human HIV-specific scTv (chapter 2) was also expressed as a fusion to intracellular signaling domains and it also mediated antigen specific T cell activity.

In chapter 4, the murine m33 scTv fusion was compared to an antibody derived chimeric antigen receptor called 237. The 237 antibody single-chain fragment variable (scFv) is specific for a tumor antigen resulting from a glycopeptide defect that is created by a mutant chaperone protein. Fusion of the 237 scFv to intracellular domains, as with the m33 scTv, mediated T cell activity against tumor cells that expressed the glycopeptide defect. Results with T cells transduced with chimeric antigen receptor in the absence or presence of coreceptor CD8, showed that CD8 could contribute to target cell recognition, presumably through its interactions with MHC molecules that are proximal to the antigen epitope.

Chapter 5 describes the engineering of a murine T cell receptor, called 3D, for enhanced affinity to the model Wilm's tumor antigen (WT-1/D^b). Using a novel T cell display system, mutated TCR libraries were displayed on the surface of T cell hybridomas in the absence of the coreceptor CD8. Selection with fluorescently labeled dimers of WT-1/D^b resulted in the isolation of two high affinity 3D TCR variants, one which mediated T cell activity in the absence of CD8. Analysis of the TCR residues used by human and murine TCRs specific for the identical WT-1 peptide suggested that there is homology between human and mouse TCR CDR3 sequences, indicating that these residues have strong selective pressures to bind to the identical peptide epitope. Thus, one may be able to engineer high-affinity TCRs in the human TCR based on knowledge from the mouse TCRs.

To My Grandparents, Helen and David Aggen and Ann and Stanley Sumner,
for Their Unequivocal Belief in Me.

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LIST OF ABBREVIATIONS

1B2 – Antibody Specific for the 2C TCR
1G4 – Human TCR Specific for NYESO/HLA.A2
2C – Murine TCR Specific for SIY/K^b, QL9/L^d
3D – Murine TCR Specific for WT-1/D^b
868 – Human TCR Specific for SL9 (Gag)/HLA.A2
A6 – Human TCR Specific for Tax/HLA.A2
AML – Acute Myeloid Leukemias
APCs – Antigen Presenting Cells
β2m – Beta-2 Microglobulin
BiTe – Bispecific T Cell Engager
BMLF1 – Epstein Barr Virus Derived Peptide GLCTLVAML
BSA – Bovine Serum Albumin
Cα – TCR Constant Domain Alpha
Cβ – TCR Constant Domain Beta
CAR – Chimeric Antigen Receptor
CDR – Complementarity Determining Region
CTLs – Cytotoxic T Lymphocytes
D^b – Murine MHC D of b
FACS – Fluorescence Activated Cell Sorting
FRET – Fluorescence Resonance Energy Transfer
GP100 – Tumor Associated Murine Peptide EGSRNQDWL
HA – Hemagglutinin
HIV – Human Immunodeficiency Virus
HLA – Human Leukocyte Antigen
HTLV – Human T Cell Lymphotrophic Virus
IL – Interleukin-2
ITAMs – Immune Tyrosine Activation Motifs
K^b – Murine MHC K of b
L – Linker
NK Cell – Natural Killer Cells
NYESO – Tumor Specific Peptide SLLMWITQC
m33 – High Affinity Murine TCR Specific for SIY/K^b
MART-1 – Melanoma Associated Peptide ELAGIGILTV
MSCV – Murine Stem Cell Virus
OTS-8 – Mutated Protein Recognized by 237 Antibody
PAMPs – Pathogen Associated Molecular Patterns
PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
pepMHC – Peptide Major Histocompatibility Complex
RBCs – Red Blood Cells
scFv – Single Chain Fragment Variable (Antibody Derived)
scTv – Single Chain T Cell Receptor Variable
SIY – SIYRYYGL Peptide

SL9 – HIV Associated Peptide Derived from GAG Protein SLYNTVATL
SPR – Surface Plasmon Resonance
Tax - HTLV Associated Peptide LLFGYPVYV
TCR – T Cell Receptor
TH1 – T Helper Cell Type I
TH2 – T Helper Cell type II
TLR – Toll-Like Receptor
Tregs – Regulatory T Cells
Vα2 – Human TCR Variable Domain Alpha 2 Gene Segment
Vβ – Human TCR Variable beta
V_H – Variable Heavy Domain (antibody)
V_L – Variable Light Domain (antibody)
WT-1 – Wilm's Tumor Antigen (Peptide: RMFPNAPYL)
WT – Wild Type

CHAPTER ONE

INTRODUCTION

A Brief Historical Overview of Immunology

The concept of immunity can be traced as far back as the 5th century B.C. in Greece when the plague raged through Athens. Thucydides recorded that a small population of individuals who had previously contracted disease were "exempt' from the current bought of plague, the first recorded instance where some individuals demonstrated protection from disease (1). It was not until centuries later that Edward Jenner developed the concept of vaccination, a way to protect from future infection by inoculation with a similar antigenic mixture (2). Jenner exposed his neighbor's son to cowpox derived from a lesion on the hand of a milkmaid and subsequently infected the young boy with small pox. The boy survived the smallpox infection because he had been previously exposed to the similar cowpox virus. Smallpox now represents a great achievement for modern vaccination, as the World Health Organization declared the virus eradicated by the 1970s (3).

Although vaccination became a common practice over the century following Jenner's work, it was not until the work of Robert Koch and Louis Pasteur that the basis of disease was elucidated. Robert Koch developed the germline basis of disease, demonstrating that microorganisms caused disease and elucidated *Mycobacterium tuberculosis* as the causative organism of tuberculosis (4). This finding allowed for the extension of Jenner's early ideas of vaccination by Louis Pasteur, who is now widely regarded as the 'parent' of immunology. Pasteur developed vaccines against cholera in chickens and rabies in humans prior to the start of the 20th century (5). Koch and Pasteur were contemporaries, but were fierce enemies during and following the outbreak of the Franco-Prussian war and there are recorded reports of Koch, provoked by Pasteur at a meeting, stating: "When I saw in the program that Monsieur Pasteur was to speak today on the attenuation of viruses, I attended the meeting eagerly, hoping to learn something new about this very interesting subject. I must confess that I have been disappointed, as there is nothing

new in the speech which Monsieur Pasteur has just made. I do not believe it would be useful to respond here to the attacks he has made on me...I will reserve my response for the pages of the medical journals" (6). Their competitive nature drove the development of the germ theory, as each raced to elucidate the causative organisms for disease.

Simultaneously with the development of the germ theory, a debate raged amongst Koch and Pasteur's contemporaries regarding immunity, with support for either a humoral (from soluble substances) or cellular (from phagocytosis) basis for protection from foreign organisms. The humoral hypothesis was led by work by Emil Von Behring and Shibasaburo Kitasuto who demonstrated that anti-toxins existed in the blood. Transfer of blood from an infected animal led to protection from infection in a separate host, an approach which they referred to as serum therapy (7),(8). It was not until much later that the substances in the serum were identified as antibodies, but this early demonstration of serum therapy resulted in Von Behring and Kitasuto receiving the first Nobel prize in physiology or medicine (9). Four years later, Robert Koch would receive the Nobel prize for his germ theory postulate (10).

Shortly after the demonstration of serum therapy, Paul Ehrlich, a German scientist, developed his side-chain theory: that side chains on cells bound to specific antigens, leading to the release of these side-chains and neutralization or eradication of foreign substances (11). Ehrlich's postulates are now considered the basis for chemotherapy and targeting of specific molecules in immunotherapy. Among Ehrlich's many accomplishments was a cure for syphilis, which was widely used prior to the introduction of penicillin.

In contrast to the humoralist approach, Ilya Metchnikoff developed a cellular hypothesis based on the ability of some species to 'engulf' other organisms (12). While studying the larvae of starfish Metchnikoff developed the phagocytosis hypothesis, that the cells of the immune system may operate in the same way that small organisms engulf other microorganisms. The macrophage immune cell type is now known to express receptors for many bacteria constituents and can phagocytose invading organisms to promote inflammation, providing a quick response to infection. The hypothesis at the time was that white blood cells took up microorganisms and

allowed septicemia to progress by spreading bacteria throughout the body. Only later would others demonstrate that quite the opposite was occurring, with white blood cells removing infectious agents. Metchnikoff and Ehrlich were awarded a Nobel prize for their contribution to immunology in 1908 (13).

The humoralist basis for immunity predominated until after World War II, when Peter Medawar began working with skin grafts. Medawar discovered that grafts from different individuals (an allograft) were rejected, while grafts of skin from the same individual to different locations were accepted (14). It was not until years later that the cellular basis of this response was understood, when James L. Gowans identified a specific blood cell type, known as the T lymphocyte, which was responsible for mediating recognition of self (syngeneic) and non-self (allogeneic) tissues (15). Thus, it was clear that there was a cellular component to immunity.

Despite these seminal experiments demonstrating a cellular, lymphocyte component to immunity, the humoral view of immunity was still widely accepted. In the mid 1950s Niels Jerne, David Talmage, and Frank Burnet developed the clonal selection theory (16), which stated that lymphocytes expressed receptors specific for antigens and that upon antigen encounter, the lymphocytes proliferated and released many receptors of a single specificity into serum to clear infection (17-19). These experiments solidified that there was a humoral (antibody) component to immunity. The humoral and T cell response to infection is today referred to as adaptive immunity. In 1960, Medawar and Burnet were awarded the Nobel prize for the discovery of acquired immunological tolerance (20) and Jerne received a Nobel prize in 1984 for theories concerning immune system development (21).

Nearly a decade after the clonal selection theory, Kohler and Milstein demonstrated the ability to produce monoclonal antibodies from cell culture (22). Around the same time, the three dimensional structure of an antibody binding fragment, called an Fab', was elucidated (23). The understanding of antibody recognition and its potential for therapeutics has led to the development of a billion dollar industry with monoclonal antibodies targeting a diverse array of antigens (24). Thus, the role of B lymphocytes in antibody production was solidified by the early 1970s.

The clonal selection theory, however, failed to explain how T lymphocytes recognize antigen. Breakthroughs in understanding the antigen recognized by T cells came from studies of tumor graft rejection in mice by George Snell (reviewed in (25)) and blood cell agglutination in transfusion by Jean Dausset (reviewed in (26)). Snell identified the murine version and Dausset the human version of what is now referred to as the major histocompatibility complex, for which both were awarded the Nobel Prize in 1980 (27).

The link between T cells and MHC protein was not clear until Rolf Zinkernagel and Peter Doherty determined that T cell recognition was restricted to MHC molecules (28). In a seminal experiment in the field of immunology, mice were infected with meningitis and their spleens were isolated. T cells from in-bred mice were able to kill the infected splenocytes, but T cell from mice of different strains were unable to kill the infected splenocytes. This demonstrated that T cells must recognize a 'self' component (MHC molecule) and a 'foreign' component (peptide), although the identity of the foreign component as a small peptide would not come until much later. Zinkernagel and Doherty were awarded the Nobel prize in 1996 for their discovery of MHC restriction in relation to T cell activity (29). It was not long after the discovery of MHC restriction that Phillipa Marrack and John Kapplar identified the T cell component of MHC recognition, the T cell receptor (TCR) (30). A number of seminal studies followed demonstrating that antibody (31, 32) and T cell receptor diversity (33) were generated by gene rearrangement. The gene sequence of a murine T cell receptor was subsequently isolated by Herman Eisen and colleagues, prompting the start of a new era probing the molecular basis of T cell antigen recognition (34, 35).

Improvements in the ability to express and crystallize recombinant protein permitted the elucidation of the structures of immunologically relevant proteins. Jack Strominger and Don Wiley solved the crystal structure of the human MHC molecule, HLA.A2, which is the most prevalent human class I MHC allele (36). This was followed by studies detailing a structure of the TCR:pepMHC complex (37). All of these studies probing the molecular basis of antibody and T cell recognition have solidified the components of the adaptive immune response. It is now well described

that the immune system has two components, a fast acting innate immune system and a slower acting adaptive immune response (T cell and B cells) that can provide immunological memory and prevent future infections.

Innate Immunity

In response to pathogens, the immune system utilizes a number of cell types such as neutrophils, macrophages, and natural killer (NK) cells that can mediate inflammation relatively rapidly, within ~4 hours of infection. The inflammatory response serves to deliver effector molecules to the site of infection, to form a physical barrier by promoting microvascular coagulation, and to increase blood flow to repair damaged tissue. Neutrophils are the most short-lived of the blood cells of the innate immune system and engulf bacteria by phagocytosis (38). The white pus often observed at a site of infection is due to the infiltration of neutrophils. In non-infectious states, neutrophils reside in the bloodstream and upon encounter with foreign substances, release chemicals and proteins of the CXCL family that promote migration to areas of infection (39). The chemokines attract more neutrophils which are also secreting proteins that can mediate inflammation called cytokines.

Macrophages are a much longer lived cell lineage compared to neutrophils and also engulf bacteria and toxins to promote inflammation (40). The macrophages secrete numerous pro-inflammatory cytokines called interleukins (IL-1, IL-6, IL-8, and IL-12) that promote inflammation and the eventual destruction of pathogens (41). More recently, a continuum of macrophage differentiation states have been identified with important roles in immune suppression collectively referred to as myeloid derived suppressor cells (42). Finally, NK cells recognize altered-self cells that lack MHC molecules through a variety of receptors and are important in the elimination of cells that are infected with virus and downmodulate MHC protein expression (43).

The cells of the innate immune system recognize specific pathogen associated molecular patterns (PAMPs) which are highly conserved among bacteria and viruses. Toll-like receptors (TLRs) are one class of receptors responsible for PAMP recognition that were initially isolated in Drosophila (44). In humans, TLRs are expressed on the surface of cells of the innate immune system, and promote the

release of cytokines as well as the upregulation of costimulatory molecules that are important for the adaptive immune response (45). For instance, dendritic cells, an important class of professional antigen presenting cells, upregulate costimulatory molecules required for T cell function upon TLR binding to bacterial or viral determinants (46). TLRs provide a critical link between innate and adaptive immunity, in that they prime the immune response for antibody and T-cell mediated responses (47). For this reason, many vaccines that consist of antigen in the form of DNA or protein are packaged with adjuvant, usually in the form of heat killed bacteria or polysaccharides to promote immunological protection. Recently, it was demonstrated that T cells also express toll-like receptors, suggesting that the TLRs may provide some benefit to effector cells by promoting release of proinflammatory cytokines (48).

Adaptive Immunity

B Cells and Antibody Response in Immunity

Antibody Diversity and Structure - Antibodies are produced by B cells in response to foreign antigens. Each antibody consists of two heavy and light chains, with each heavy and light chain containing variable and constant domains. The variable-heavy (V_H) and variable-light (V_L) domains mediate antigen recognition, with each antibody containing two V_H-V_L heterodimers (Figure 1.1A). The remarkable diversity of the antibody repertoire (estimated on the order of 10^{11} unique receptors) is a result of 1) combinations of V_H and V_L gene segments, 2) somatic gene rearrangements that occur during development, and 3) somatic hypermutation that occurs in peripheral lymphoid organs (49). The true size of the antibody repertoire, however, is dependent on the number of mature B cells in an individual which is estimated to be between 10^8 and 10^9 B cells .

B Cell Development: B cells mature in the bone marrow, where they encounter soluble and cell bound self-derived antigens (50). B cells expressing membrane bound antibodies that bind to self tissue either become anergic (inactive) or continue gene rearrangement until an antibody is expressed that does not bind to self-tissue. The B cells expressing cell-bound versions of these antibodies escape into

the periphery, and upon foreign antigen encounter, a single B cell expressing a single antibody proliferates, producing soluble antibody molecules containing various fragment crystallizable (Fc) regions (Figure 1.1A) (51). Naive B cells produce IgM isotype antibodies, and following interaction of B cells with helper $CD4^+$ T cells, class switching to other isotypes such as IgG occurs (52). Upon initial antigen encounter, B cells also differentiate into a memory cell population which provides protection and fast-acting responses upon second encounter with the same antigen (53). Generation of the B cell memory population is a goal of current vaccination strategies to provide long-term host immunity.

The term antibody Fc originates from initial experiments where antibody digestion with pepsin yielded two fragment antigen binding (Fab) molecules and one Fc region (Figure 1.1A). The fragment antigen binding has been further modified, to create antibody single chain fragment variable proteins (scFv), consisting of the V_H and V_L region of an antibody Fab attached by a flexible linker, for therapeutic applications and antibody biophysical characterization (Figure 1.1B). For instance, antibody scFv directed against tumor antigens have been fused to anti-CD3 antibodies, to promote T cell activation of T cells near cancer cells (54). The Fc region of soluble antibodies determines the antibody isotype; specific antibody isotypes (IgG,IgA,IgE) can bind to Fc receptors on the surface of various immune cells (i.e. NK cells) which can result in phagocytosis of pathogens bound to antibodies or activation of immune cells to secrete proinflammatory cytokines (55-57). A second mechanism of antibody action involves the binding of soluble proteins collectively called complement to antigen:antibody complexes (58). A cascade of complement proteins direct the destruction of antigen or phagocytosis of 'foreign' substances. B cell activation, however, often requires priming by helper $CD4^+$ T cells of the adaptive immune system.

T Cells

Classes of T Cells. Two classes of T cells that express αβ full-length TCRs mediate immunity and are defined based on their coreceptor expression (59). Expression of the coreceptor CD8 is associated with recognition of class I

peptide:MHC molecules, and $CD8^+$ T cells are referred to as cytotoxic T lymphocytes (CTLs) because these cells mediate lysis of target cells expressing cognate pepMHC. CD8 acts as a coreceptor to bind to an invariant region of the α3 domain of MHC molecules (60) and improves class I restricted TCR sensitivity to antigen by recruiting kinases, such as LCK, to the T cell receptor signaling complex, initiating T cell activation (61). Upon antigen encounter, $CD8^+$ T cells release a variety of cytokines, including interleukin-2, that allow for expansion of T cells of the same specificity, a process called clonal selection. The CTL releases granzymes and perforins that ultimately cause lysis of the pepMHC expressing target cell (62).

A second class of αβ TCR expressing cells, called $CD4^+$ (helper) T cells, recognize peptides in the context of MHC class II molecules. The coreceptor CD4 binds to nonpolymorphic regions of the class II MHC, stabilizing the TCR:pepMHC interaction and initiating T cell signaling (63,64). TCR binding results in differentiation of the $CD4^+$ T cells into Th1, Th2, and Th17 cell types, with differentiation determined by interaction with other immune system cells and the local cytokine environment (65). For instance, the presence of IL-12 and the absence of IL-4 leads to differentiation of $CD4^+$ T cells to the Th1 fate (66). Th1 cells are specialized for macrophage activation, permitting macrophages to release cytokines and other mediators of the immune response (67). Th2 cells primarily activate B cells, and contribute to the humoral, antibody response to infection. Th17 cells are a recently defined subset of CD4+ T cells that are important for mediating immunity at mucosal barriers, and excessive differentiation into the Th17 phenotype is thought to play a role in autoimmune disease (68). $CD4^+$ T cells can prime $CD8^+$ T cells for activation, through $CD4^+$ T cell interaction with dendritic cells (69). $CD4^+$ T cells express CD40L (ligand) upon T cell activation and bind to CD40 on dendritic cells, which can subsequently prime a $CD8^+$ T cell response (70,71). A fourth class of CD4+ T cell, with the phenotype $CD4^+$,$CD25^+$, $FOXP3^+$ are referred to as regulatory T cells and represent another newly defined class of T cells that serve as a safe-guard to prevent autoimmunity and maintain peripheral tolerance (72).

Other T cell lineages exist that express a different class of TCR, called γδ T cells, comprise <5% of the total T cell population (73). Unlike αβ TCR positive T

cells, γδ T cells recognize non-classical MHC molecules, such as the glycopeptide CD1c, and these cells are thought to provide protection in the gut and other mucosal barriers. The γδ lineage of T cells also demonstrates some components of innate immunity, in that these cells can perform phagocytosis and recognize cellular stress signals including pathogen associated molecular patterns (PAMPs) through TLRs. Nonetheless, the γδ T cell lineage undergoes gene rearrangement like αβ TCRs, and thus is considered a component of the adaptive immune system despite their innate like functions.

Development of αβ T Cells. T cell recognition is restricted to MHC antigens during development in the thymus (74). Different combinations of variable α and β gene segments create diversity among αβ TCRs. A conservative estimate suggests that in human TCRs there are at least 1400 combinations of TCR α and β chains. An additional source of TCR diversity is recombination of variable region TCR genes (V-J or V-D-J recombination), similar to the recombination events that occur in the generation of antibodies. Cells initially start as double negative ($CD4^-$ $CD8^-$) thymocytes, and rearrange alpha and beta TCR gene loci until a productive αβ TCR pair is formed. T cells then progress to a double positive ($CD4^+$ $CD8^+$) fate. To receive a survival signal, αβ TCRs must recognize a self-pepMHC. This process of restricting T cells to pepMHC antigens is called positive selection, and assures that T cells recognize antigens in the context of self-MHC proteins (75).

The presence of coreceptor enhances binding affinity to either class I or class II MHC, and T cells progress to either a $CD4^+$ or $CD8^+$ fate (76). A small percentage of cells may escape as double positive cells, but this percentage is quite small relative to the number of cells that escape as single-positive $CD4^+$ or $CD8^+$ cells. A transcription factor, called AIRE, allows for the transcription of nearly every self protein such that peptides derived from all 'self' protein are expressed in the context of MHC proteins (77). Subsequently, TCRs that bind with too high affinity to self pepMHC undergo cell death, a process called negative selection (78). As a consequence, the affinity of most TCR:pepMHC interactions is quite low (K_D=1-100 μM). This thymic selection process is crucial to assure that T cells do not escape into

the periphery that are capable of tight binding to self-pepMHC, which would ultimately result in T cell mediated autoimmunity. For instance, T cells specific for myelin basic protein:MHC complexes have been implicated in multiple sclerosis disease progression (79). The T cells that cause multiple sclerosis are thought to have escaped negative selection (central tolerance), and avoid peripheral T cell tolerance mechanisms following T cell expansion from exposure to microbial peptides that mimic peptides derived from MBP. The T cells that proliferated in response to bacterial infection then react with myelin basic protein peptides in the context of MHC, resulting in autoimmune destruction of the central nervous system.

Generation of a T Cell Memory Population. Prior to foreign antigen encounter, it is estimated that the initial T cell population has roughly 1 in 100,000 T cells specific for a given foreign antigen (80). After antigen exposure, there is a rapid expansion of T cells specific for a given antigen (estimated to be 50,000-fold in number) (81). Once infection has resolved, the majority of T cells specific for the foreign antigen undergo apoptosis, and a residual 'memory' cell population of T cells survives such that upon second encounter with the identical antigen, infection is easily cleared (82). In the case of smallpox in humans, vaccination has been shown to provide T cell memory for as long as 75 years (83).

Molecular Basis for αβ T Cell Antigen Recognition

T Cell Receptor Proteins. Recognition of antigenic pepMHC leads to lysis of target cells and the rapid expansion of clonal T cell populations that ultimately eliminate infected or transformed cells expressing 'foreign' antigens. Recognition of these 'foreign' antigens, in the form of non-self peptide in the context of self MHC proteins, is mediated by the αβ T cell receptor (84). The TCR is an αβ heterodimer, most analogous to a Fab fragment, with each chain containing a membrane proximal constant domain and a membrane distal variable domain (Figure 1.1C) (85). The variable domains of the α and β chain mediate recognition of pepMHC. Within the variable domains six complementarity determining regions (CDR) or loops of the TCR contribute the majority of the binding energy for interaction with peptide-MHC. Because the TCR variable domains mediate antigen recognition, our lab has

previously developed a single-chain TCR variable (scTv) format (Figure 1.1D) for engineering and protein expression, consisting of the TCR variable domains attached by a flexible linker (86). The scTv receptor format provides a potential advantage for TCR engineering in that yeast surface display levels of scTv are higher than full-length TCR. As a potential therapeutic the scTv also provides potential advantages, in that the scTv might have improved tissue penetration relative to soluble full-length TCR or cell mediated targeting strategies.

Major Histocompatibility Complex (MHC) Proteins. The major histocompatibility complex protein is one of the most polymorphic proteins known, with more than 1500 variants for the human class I allele currently elucidated (87). The MHC proteins are also polygenic, meaning that each individual usually expresses three different class I MHC alleles and three different class II MHC alleles. Two classes of MHC proteins present to T cells: class I MHC proteins present peptides between 8 to 11 amino acids in size to CD8+ T cells and these MHC proteins are expressed on all nucleated cells in the body (88), whereas class II MHC proteins present longer peptides of between 12-19 amino acids to CD4+ T cells and are only expressed on certain professional antigen presenting cell lineages. Notably, class I MHC present peptides generated by proteasome mediated destruction of intracellular proteins (class I MHC) (89). In contrast, class II MHC present peptides derived from endocytic vesicles in macrophages, B cells and dendritic cells (class II MHC) (90). This allows T cells to mount a response against extracellular pathogens that are phagocytosed in the periphery.

The crystal structure of numerous class I pepMHC proteins has revealed that the class I binding peptides are often bulged at the center, with residues in the center of the peptide more TCR exposed (91). Class I peptides generally have N and C terminal peptide anchor residues in close contact with the MHC (92). Our lab has previously characterized class I MHC peptide binding variants, and demonstrated that mutations at nearly any position of the peptide will have some effect on TCR or MHC binding (93),(94). The class II MHC, in contrast, presents longer peptides with less characteristic bulge directed at the TCR (95). Thus, there are certain constraints for peptide binding to different MHC alleles to assure surface lifetime of peptide MHC is

sufficient to mediate a T cell response (i.e. weak binding peptides may be less immunogenic). The requirement for MHC restriction in T cell response allows for T cell targeting of intracellular and extracellular pathogens (uptake by MHC Class II), as opposed to antibody mediated responses that are often directed against primarily cell surface antigens.

Structure of the T Cell Receptor/pepMHC Complex: The interaction of pepMHC with TCRs has been characterized by structural, thermodynamic, and kinetic studies. The structures of numerous TCR/pepMHC complexes have shown that the TCR engages class I pepMHC in a diagonal orientation (Reviewed in (59)). TCR complementarity determining regions 3 (CDR3) interact largely with the peptide, while CDR1 and CDR2 are focused toward the MHC (96),(97). Flexibility of the TCR CDR loops and the diversity of the T cell population (~10^8-10^{10} unique TCRs limited by the number of naive T cells in an individual) permits cross-reactivity with other peptides, allowing for recognition of a diverse array of non-self (foreign) antigens (98, 99).

Relationship Between TCR:pepMHC Affinity and T Cell Activity. If binding between T cell receptor and pepMHC is of sufficient affinity, T cells enter various states of activation resulting in death of the antigen presenting cell (APC) and proliferation of the antigen-specific T cell (100). Studies performed by our lab using TCRs engineered with greater than 1000-fold increases in affinity relative to the wild-type TCR for foreign pepMHC (SIY/K^b) showed that improved affinity TCRs could mediate enhanced antigen sensitivity (IL-2 release) (101). Our lab has now extensively characterized the binding parameters for murine TCR:pepMHC interactions, and defined a threshold delineating CD8 independent T cell activation. TCRs with affinities lower than K_D= 1 μM can mediate T cell activation independent of the coreceptor CD8 (102). Engineering of receptors with affinities below this threshold could provide potential advantages for experimental therapeutics because class I restricted TCRs could be introduced into $CD4^+$ T cells to redirect a helper T cell response.

T Cell Signaling. T cell activation is thought to require two signals: signal 1 is transmitted through T cell receptor (TCR) recognition of pepMHC and signal 2 is

mediated through costimulatory molecules on the T cell that recognize specific ligands on APCs, such as CD28 on T cells interacting with ligand B7 on APCs (103). The αβ TCR must assemble with other proteins, known as CD3 proteins, at the surface of T cells to mediate signal 1. Biochemical studies have now identified that the minimal T cell signaling subunit consists of an αβ TCR with two heterodimers of CD3δε and CD3γε and a homodimer of CD3ζζ (Figure 1.2A) (104). The CD3ζ subunits contain immune tyrosine activation motifs (ITAMs) which are phosphorylated upon T cell receptor engagement, and phosphorylation of these tyrosine motifs leads to downstream signaling events that mediate T cell activation (105). Although it remains unclear how the full-length TCR transmits signals from the extracellular environment to mediate T cell activity, two predominant hypotheses exist. The first proposes that a conformational change occurs upon pepMHC binding within the TCR constant domains that allows ITAMS to be more accessible (106). The alternative hypothesis supports a model of T cell receptor clustering, whereby engagement of multiple TCRs in close proximity leads to antigen signaling (107). Although the exact mechanism of TCR mediated signaling remains unclear evidence exists that an intracellular kinase, called LCK, interacts with ITAMs on CD3 proteins as one of the initial events in TCR signaling (108). The coreceptor CD8 is known to sequester LCK to the immunological synapse and lower the barrier for T cell activation improving T cell sensitivity to antigen (61). Another cell surface molecule, CD45, regulates LCK activity and is not incorporated into lipid rafts near the T cell receptor providing a way to regulate T cell activity (109, 110). The numerous components required for T cell activity assure that there is not aberrant T cell reactivity with self antigens.

Regulatory Mechanisms to Dampen the Immune Response. Given the multiple ways in which the immune system can generate proinflammatory responses, a number of safeguards have evolved to prevent autoimmunity. Excessive stimulation through CD28 leads to upregulation of CTLA-4 on $CD4^+$ T cells, and CTLA-4 binds to B7 on APCs and inhibits T cell activation (111). A new class of T cells has also been identified that secrete inhibitory cytokines, known as regulatory T cells (Tregs), that suppress $CD4^+$ and $CD8^+$ T cell activity. A continuum of various states of

macrophage differentiation, referred to a myeloid derived suppressor cells (MDSCs) that are $CD11b^+$, $GR1^+$ also are thought to suppress T cell function (42, 112). In disease states where the immune system is insufficient to provide host protection (i.e. T cell tolerance of cancerous tissue), therapies that inhibit these immune modulators may provide clinical benefit. For instance, clinical trials are underway for various malignancies to evaluate anti-CTLA-4 antibodies to prevent T cell anergy (113). In the setting of transplant tolerance and autoimmune disease, however, it would be advantageous to stimulate these immune regulatory cells (i.e. MDSCs could aid in tolerance of foreign tissue in transplantation) (114).

Immune Response In Organ Transplantation (Allografts)

The T cell receptor is integral in discrimination of self and non-self tissue. Individuals who have had organ transplants, even from MHC-matched (HLA) donors must take immunosuppressive medications to avoid host vs. graft disease and rejection of transplanted tissue, predominantly from host T cells binding to transplanted tissue and mounting an immune response. It has been reported that >10% of T cells can react with foreign pepMHC molecules, but the molecular basis for this reactivity has not been fully explained (115).

Two views of TCR recognition of allogeneic (foreign) MHC propose that either the TCR recognizes polymorphisms present among the foreign MHC allele with limited peptide recognition (116), or rather, that the TCR recognizes evolutionarily conserved residues between the foreign and self MHC molecule, with binding energy focused predominantly on the peptide (117). Elucidating which binding orientations predominate for allo-MHC recognition has been limited by the fact that relatively few allogeneic peptide ligands have been identified, and difficulties in expressing and crystallizing TCRs. Using yeast display, our lab was able to engineer a stabilized scTv version of the murine 2C TCR (118). Subsequently, the structure of the murine 2C scTv with both self and foreign pepMHC demonstrated that the TCR binds pepMHC in a similar docking orientation, but uses different conserved residues to recognize foreign ($QL9/L^d$, allogeneic) and self ($DEV8/K^b$, syngeneic) MHC (97). The implication of this study was that both

peptide and MHC-centric contacts with TCR mediate cross-reactivity. More crystal structures, however, with self and foreign MHC ligands need to be elucidated to determine if this docking mode can be generalized to other foreign MHC interactions, which ultimately should provide insight into the molecular mechanisms of allo-recognition.

Crystallization of the 2C scTCRs in complex with cognate and allogeneic pepMHC was only made possible through the production of soluble αβ single-chain TCRs (scTvs) engineered by yeast display. Difficulties in expressing soluble forms of other TCRs have limited analysis of T cell allorecognition, and, as a consequence, strategies are needed to improve TCR production as soluble molecules.

Immune Response to Cancer

Multiple studies have now demonstrated the importance of T cells in the elimination of human cancers. For example, the importance of a T cell response was implied in recent analyses of ovarian carcinomas (119), breast carcinomas (120), renal carcinomas (121), and in studies involving the adoptive transfer of *ex vivo* expanded T cells in melanoma (122,123,124,125), and breast cancer (126) patients. In the ovarian cancer study, the five year survival rate of patients with tumor infiltrating T cells was 83%, in comparison to a survival rate of 44.5% in patients who failed to have significant T cell infiltration (119). Intratumoral T cell infiltration has been strongly correlated to MHC class 1 surface expression in melanoma patients, suggesting that CD8+ cytotoxic T lymphocytes (CTLs) are central to cancer elimination (127). Numerous tumor antigens in the form of specific pepMHC complexes have now been defined allowing for the generation of T cell receptors of defined specificity to redirect T cells towards cancerous tissue.

One promising approach to tumor elimination involves the use of adoptively transferred T cells directed against tumor cells. The regression of tumors using an adoptive immunotherapy approach has been demonstrated successfully subsequent to lymphodepletion in melanoma patients (128-130) and the persistence of T cell populations specific for cancerous tissue has been observed one month after transfer (128). Adoptive immunotherapy strategies provide a route to target cancer tissue

specifically, while generating a T cell memory population that could eliminate residual disease. Because CTLs against "self" peptides are deleted in the thymus during T cell development, individuals may be left with a T cell repertoire that is unable to eliminate the cancer tissue. The absence of these T cells represents a major problem with current T cell based strategies: *an individual may lack appropriate anti-tumor T cells due to tolerance mechanisms that operate against self-antigens*.

To overcome the lack of tumor specific T cells, gene transfer of T cell receptors *ex vivo* provides an attractive alternative. T cells specific for tumor associated antigens (TAAs) can be generated from the peripheral blood of healthy donors, and isolation of TCR genes specific for these TAAs allows for use in many patients. Thus, a high affinity TCR specific for tumor associated pepMHC can be introduced into patients' $CD4^+$ or $CD8^+$ T cells *in vitro*, and transfer of transduced T cells can eradicate tumors (Figure 1.3). Regression has been observed in as high as 30% of patients following adoptive transfer of TCR gene modified T cells (131, 132). Recently, TCR gene transfer with an adoptive transfer protocol was used to eradicate metastatic melanoma in a small subset of patients in the brain (133). Adoptive therapy clinical trials using TCR gene therapy are ongoing for melanoma, but it is unclear if the TCRs selected are of optimal affinity for targeting tumor antigen. The TCR gene therapy approach holds promise for treating a variety of tumors provided receptors can be engineered with sufficient affinity for tumor antigen. Our lab and others have previously engineered T cell receptors for high affinity, but there are still relatively few *in vitro* engineered human, high affinity TCRs.

Challenges for Current T Cell Adoptive Therapies. In TCR gene therapy based approaches, the lack of many high affinity receptors limits the possible antigens and tumor types that can be targeted. While multiple studies at the NIH have explored targeting the melanoma antigens MART-1 and GP100 (132), the *in vitro* binding affinities of these receptors have not been measured by biochemical methods. There are still a multitude of other tumor associated pepMHC antigens derived from either mutated self or overexpressed self proteins that need to be evaluated for TCR gene therapy (listed in (134). Clearly, development of straightforward methods to engineer

TCRs derived from tumor specific T cell clones would be of great value, and this is a focus of chapters 2 and 5 of this thesis.

TCR gene transfer is also potentially dangerous in that introduced alpha and beta TCR chains can mispair with endogenous TCR chains to create mismatched heterodimers of unknown specificity, leading to autoimmunity (Figure 1.3B) (135, 136). Strategies that eliminate mispairing between introduced and endogenous TCRs need to be evaluated to avoid autoimmunity from these mismatched receptors. A variety of strategies have been explored to prevent mispairing, but the extent to which these strategies prevent mispairing has not been elucidated (137). It is important to develop TCR reagents that can recognize MHC-restricted antigens, in addition to antibodies against cell surface antigens, to ensure that support cells, collectively called tumor stroma, are targeted. Studies have demonstrated that stromal cells can take up peptide antigen and cross-present tumor associated peptides on MHC molecules (138, 139). Targeting of tumor stroma may be necessary to destroy solid tumors, and antigens cross-presented on tumor stroma are potentially neglected by antibody based therapies (140).

Monoclonal Antibody Based Therapeutics. - Another approach to tumor immunotherapy utilizes monoclonal antibodies specific for tumor associated proteins. In the mid-2000s, there were at least 400 monoclonal antibodies in clinical trials with specificity for a variety of tumor antigens (141). Although the exact mechanisms of monoclonal antibody destruction of tumors remain elusive, it is thought that soluble antibody binding to tumor cells marks tumor cells for antibody dependent cell cytotoxicity or promotes activation of other immune cells in the tumor microenvironment.

Some tumor cells express low levels of antigen and as a consequence, soluble antibodies are inefficient in destroying tumor tissue. To improve response to low levels of antigen, numerous groups have developed chimeric antigen receptors (CARs). The scFv CARs combine TCR activation signal 1 and signal 2 on one molecule, and, when endowed with the appropriate intracellular signaling domains, can operate independently of the full-length TCR signaling subunits (Figure 1.2B) (142, 143). Transduction of CAR constructs allows for the redirection of T cell

mediated responses to tumor tissue in adoptive therapy formats. The CAR constructs usually consist of a single-chain fragment variable (scFv) fused to intracellular signaling molecules. The scFv is derived from the variable domains of an antibody attached by a flexible linker, while the intracellular signaling subunits almost exclusively include the CD3ζ subunit. Second generation constructs contain additional intracellular signaling subunits that promote T cell costimulation, such as CD28, CD3ζ, LCK or CD28, 4-1BB, CD3ζ (144). Antibody CAR based approaches are potentially limited by their inability to target tumor stroma, since antibodies often target cell surface proteins that are not cross-presented.

Engineering T Cell Receptors *in vitro* For Improved Stability and Affinity

Difficulties in producing soluble TCRs, relative to antibodies, have limited the ability to characterize the binding affinities and cross-reactivities of TCRs for specific pepMHC. The challenges of producing T cell receptors are highlighted by the relatively few TCR:MHC crystal structures solved to date relative to antibodies. As of 2004, less than 30 murine and human structures of the TCR and pepMHC in complex had been solved (59), whereas a conservative estimate for antibodies suggest that by 2004 more than 500 antibody structures had been elucidated (145).

Previously, our lab has developed a single-chain TCR format, consisting of only the TCR binding domains attached by a flexible linker (Vα-L-Vβ or Vβ-L-Vα) (reviewed in (146). We have termed these receptors single-chain TCR-variable, by analogy but distinct from antibody scFv. The ability to engineer TCRs *in vitro* is important, since the process of negative selection leaves a repertoire of T cells with relatively low affinity for pepMHC (K_D=1-100 μM). Our lab has previously developed two systems, yeast display and T cell display (147), to engineer T cell receptors for enhanced affinity for pepMHC (Figure 1.4). In the course of engineering a number of T cell receptors, we have found that not all TCRs are amenable to engineering by yeast display (86) and hence, have also used a T cell display platform for engineering. Others have used phage display for isolation of high affinity TCRs as full-length receptors (148-150), but to date there are still relatively few *in vitro* engineered human, high affinity TCRs.

High affinity TCRs have potential immunotherapy applications as both soluble and cell mediated therapies for targeting pepMHC antigens. Soluble TCRs could be used in therapeutic applications, to potentially deliver a toxic payload to tumor cells overexpressing antigen (in the form of pepMHC) or in diagnostic applications to identify tissues overexpressing tumor or virus associated pepMHC molecules. In cell mediated applications, high affinity TCRs could be introduced into patients using TCR gene therapy (Figure 1.3). *In vitro* studies with high affinity murine TCRs have demonstrated that improving affinity beyond a certain threshold confers CD8 independence, allowing class I restricted TCRs to have enhanced sensitivity to antigen in response to foreign pepMHC, and the potential to redirect CD4+ T cells to class I restricted antigens (147,151).

Overview of Thesis Work

Understanding the molecular basis of T cell receptor recognition has been severely limited by difficulties in expressing and crystallizing soluble forms of T cell receptors. Because variable domains of the T cell receptor are all that is required to mediate recognition of specific pepMHC, our lab has extensively developed scTv molecules to study T cell receptor interaction with pepMHC. Since the scTv molecules are unstable in the absence of TCR constant domains, mutations must be generated that permit expression of the scTv. Yeast surface display of mutant scTvs has allowed for screening and isolation of TCRs of improved stability, with surface display correlated with soluble secretion efficiency and thermal stability (152). Subsequent production of soluble versions of stabilized scTv fragments permitted analysis of the binding properties of soluble TCRs to cognate pepMHC by surface plasmon resonance, as well as crystallization of single-chain TCRs to assess cross-reactive among syngeneic and allogeneic MHC recognition, which is of great importance for understanding transplant rejection.

The stabilized scTv templates were also used to generate high affinity T cell receptors *in vitro* using yeast display. High affinity variants of the murine $CD8^+$ 2C TCR specific for pepMHC molecules SIY/K^b (101) and QL9/L^d (153) and $CD4^+$ 3.L2 TCR specific for the pepMHC molecule Hb/I-E^k (154) have been isolated and

characterized. When the high affinity receptors were reintroduced into T cells as full-length receptors, in all cases improvements in T cell affinity enhanced T cell activity in response to low levels of antigen.

Successes with yeast display, however, have previously been limited to murine T cell receptors that had antibodies specific for the properly folded Vα and Vβ domains of the scTv that permitted isolation of stable scTv templates for engineering. To translate these findings into human TCR systems, the goal of this thesis work was to engineer human T cell receptors in a single-chain format for immunotherapy applications. Subsequently, I sought to develop ways to utilize single-chain TCRs in the context of T cell mediated targeting therapy, by creation of scTv fusions to transmembrane and intracellular proteins referred to as chimeric antigen receptors (CARs). I compared the efficacy of CARs with scFv or scTv extracellular binding domains in targeting tumor cells and peptide pulsed cells. Finally, I extended another engineering platform, known as T cell display, to the engineering of a murine T cell receptor against a model tumor antigen.

In chapter 2, the engineering of improved stability variants of two human TCRs specific for peptides derived from human T cell lymphotrophic virus (HTLV) and human immunodeficiency virus (HIV) is described. These receptors were previously engineered by phage display in a full-length format for enhanced affinity (148, 149), allowing for selection of stabilized scTvs using pepMHC streptavidin:phycoerythrin tetramers. Notably, both of the engineered human receptors utilize the same human Vα2 gene segment (IMGT nomenclature: TRAV12-2). Evaluation of the improved stability version of the HIV-specific receptor, called 868, demonstrated that few Vα mutations were required for yeast surface display and production as inclusion bodies in *E. coli*, suggesting that other human Vα2 gene segment may be amenable to engineering in an scTv format using yeast display. Production of other soluble scTv molecules should allow for measurement of wild-type TCR binding affinity, as well as the generation of scTvs of enhanced affinity against a wide variety of clinically relevant human antigens. The ability to engineer human TCRs should allow for future analysis of T cell receptor cross-reactivity to improve understanding of self vs. nonself discrimination. The soluble, human 868 scTv was also used for detection of

low nanomolar concentrations of HIV-specific peptide on the surface of target cells, demonstrating a diagnostic application of the scTv.

Previously, our lab has established single-chain, high affinity versions of the murine 2C TCR called m33 (101). In chapter 3, chimeric antigen receptors consisting of 2C (low affinity) or m33 (high affinity) scTv fused to the intracellular signaling molecules CD28, CD3ζ, and LCK were evaluated. The scTv constructs avoid mispairing with endogenous TCRs, which has been shown in murine (135) and human systems (136) to cause autoimmunity through generation of receptors of unknown specificity. These receptors provide a potential advantage relative to other CAR constructs in that they may be able to target tumor stroma cross-presenting pepMHC. I compared m33 full-length TCR to m33 scTv for mediating T cell cytokine release and demonstrate that stabilized scTv can be introduce into CAR constructs to redirect T cells to antigen. I also extended this study to the HIV-specific 868-z11 scTv engineered for improved stability by yeast display (chapter 2), and demonstrate that a human scTv can mediate antigen specific T cell activity in murine $58^{-/-}$ T cells.

A number of chimeric antigen receptors derived from antibody scFv are currently being explored to target a variety of tumors (155). In chapter 4, I created a scFv chimeric antigen receptor derived from an antibody, called 237, isolated by Professor Hans Schreiber's Lab (U. of Chicago) that recognizes a mutated glycopeptide present only on tumor cells. The 237 scFv CAR contained CD28,CD3ζ,and LCK intracellular subunits analogous to the m33 scTv CAR described in chapter 3. The 237 scFv CAR mediated T cell activity in response to tumor cells expressing mutated glycopeptide, but not tumor cells that have been transfected with a chaperone that repairs the glycosylation defect. I propose a model for CD8 contribution to scFv CAR activity, where CD8 coadhesion to MHC molecules that neighbor 237 glycopeptide epitopes aid in generating T cell activation. This finding has implications for CAR-mediated activity directed against tumor cells, in that tumor cells can downmodulate MHC molecules to evade the immune response, and in these instances, CAR constructs introduced into $CD4^{+}$ T cells may provide less potent responses. Chapter 4 also describes the modification of the original m33 scTv CD28,

CD3ζ, LCK construct to contain the 4-1BB intracellular signaling domain, which has been shown by others to improve CAR-transduced T cell persistence *in vivo* (144). A construct consisting of m33 scTv fused to CD28, 4-1BB, and CD3ζ was compared with the LCK containing m33 scTv and the m33 full-length TCR. I describe the requirement of endogenous LCK and LCK attached to the CAR construct in promoting T cell cytokine release.

In the final chapter, the engineering of two improved affinity variants of a murine TCR, called 3D, specific for the model tumor antigen WT-1/D^b is described. Difficulties in creating stable platforms to engineer enhanced affinity T cell receptors led us to explore a T cell display platform that was used to isolate an improved affinity variant of the 2C TCR (147). Using a similar strategy, two higher affinity variants of the 3D TCR were isolated, one of which permitted CD8-independent activation. Professor Phillip Greenberg's lab (U. of Washington) has isolated a number of human WT-1 specific TCRs that recognize an identical peptide to the murine 3D TCR in the context of a human MHC. Homology was observed in a number of the CDR loops, suggesting that there may be some conserved interactions between murine and human TCRs in contacting a self-peptide. The 3D high affinity variants provide valuable reagents for studying the affinity thresholds that dictate peripheral and central tolerance. These TCRs should also allow for the development of an adoptive therapy model system to study the optimal affinity TCR range for targeting overexpressed self-antigen, providing maximal T cell function while limiting autoimmunity directed against non-malignant tissues.

Figures

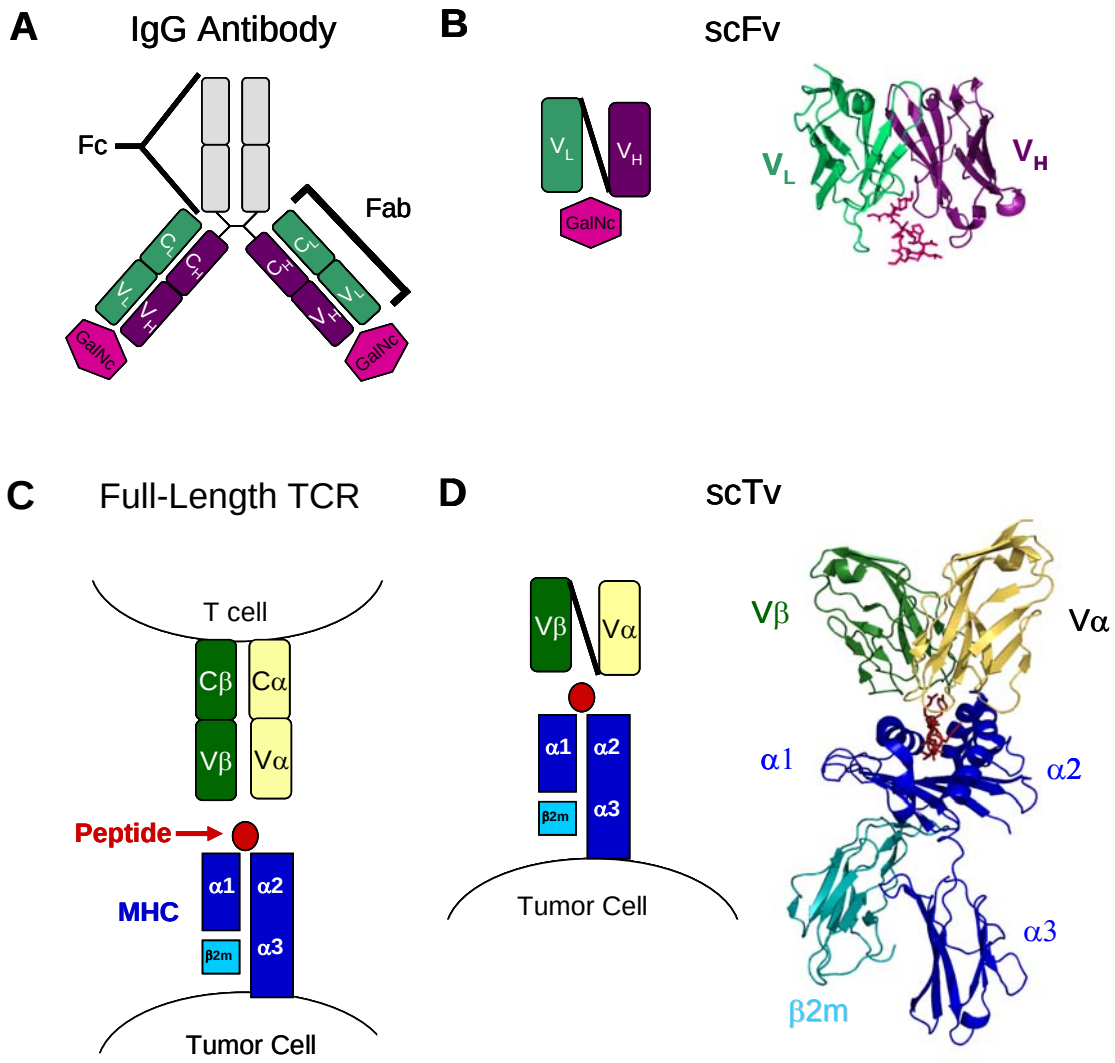

FIGURE 1.1 Formats of T Cell Receptors and Antibodies. (A) Schematic of an IgG antibody, with fragments resulting from pepsin digestion as indicated. Fab=Fragment Antigen Binding, Fc=Fragment Crystallizable, V_H=variable heavy domain, V_L= variable light domain, C_H=constant heavy domain, C_L= constant light domain, GalNc=synthetic peptide antigen. **(B)** Schematic and crystal structure of 237 scFv in complex with GalNc (Magenta) synthetic glycopeptide (PDB: 3IET) (259). **(C)** Schematic of an αβ T Cell receptor binding to pepMHC. TCR domains: Vα=Variable alpha, Variable beta, Cα=Constant alpha, Cβ=Constant beta. (D) Schematic and crystal structure of the 2C TCR (shown as scTv) with SIY/K^b (PDB: 1G6R) (91).

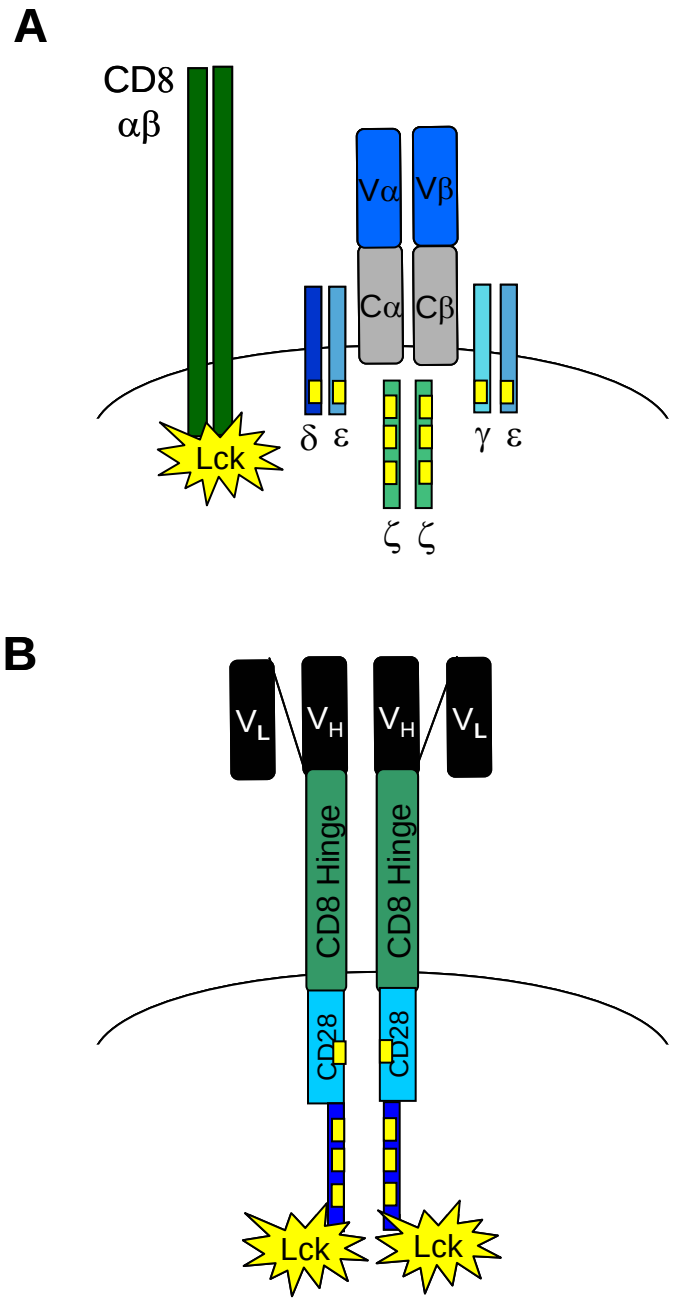

Figure 1.2. T Cell Receptor and Chimeric Antigen Receptor Complexes. (A) Schematic of the T cell receptor signaling complex with associated CD3 subunits. Yellow squares= immune tyrosine activation motifs (ITAMs). CD8 coreceptor binds to the intracellular kinase, Lck (yellow starburst), sequestering the kinase to the CD3 subunits. **(B)** Schematic of two chimeric antigen receptor that can mediate T cell activity independently of the endogenous CD3 subunits.

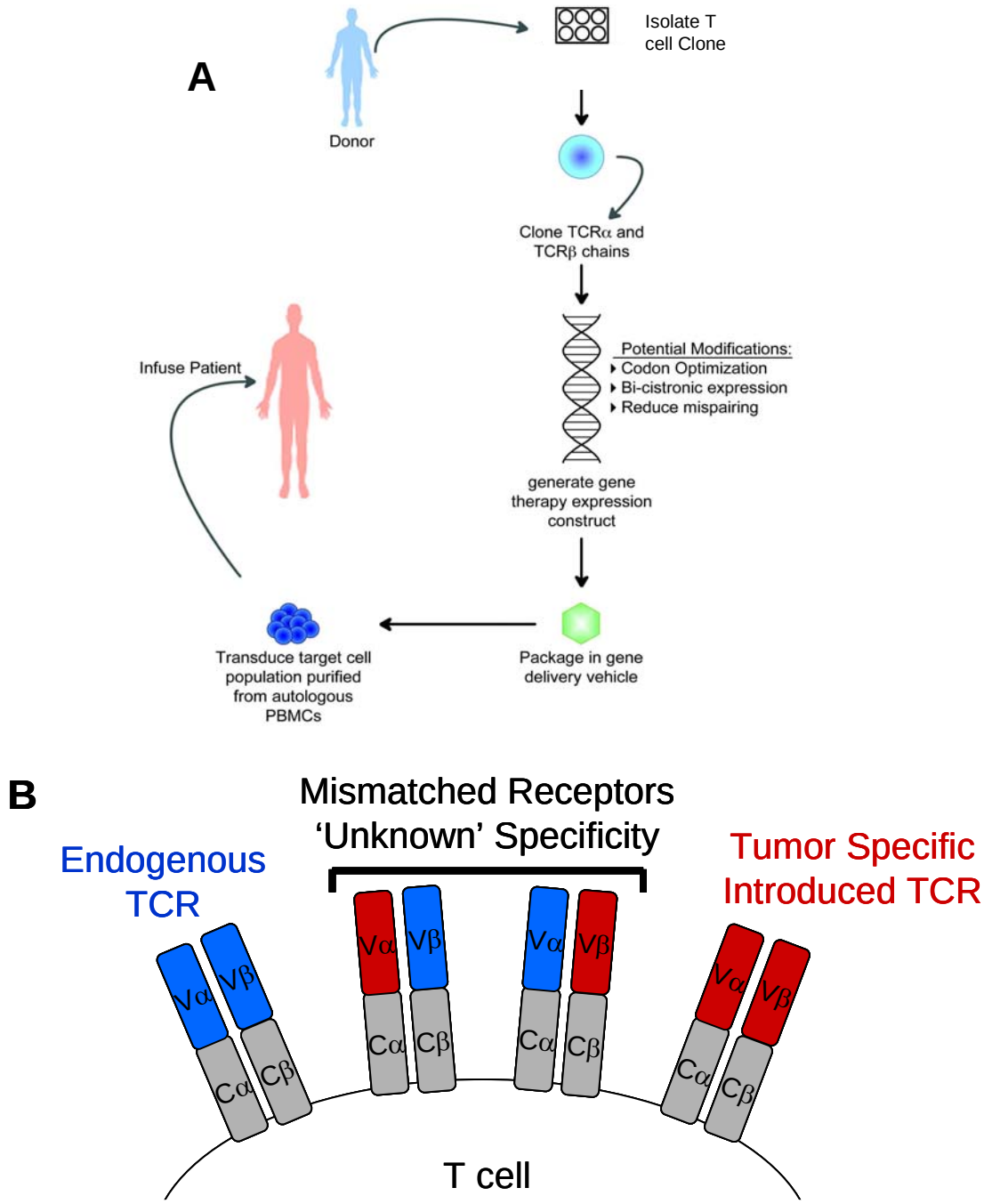

Figure 1.3. Overview of Adoptive T Cell Therapy with TCR Gene Transfer, and Potential for Generation of Receptors of Unknown Specificity. (A) An overview of TCR gene therapy. Green = Retroviral supernatent containing TCR genes. Blue = T cells expanded from patient (pink). Figure (A) from Schmitt et al. (197). reproduced with permission from Mary Ann Liebert publications. **(B)** TCR gene transfer into host T cells can either involve pairing between introduced α and β chains, or can cause creation of mismatched receptors between introduced (tumor-specific) and endogenous TCR, potentially leading to autoimmunity.

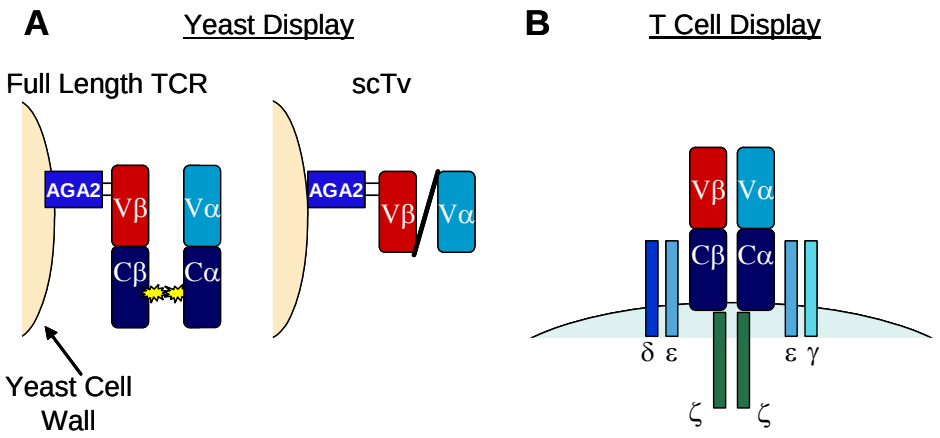

Figure 1.4. Yeast and T cell Display Platforms for TCR Engineering. **(A)** Yeast display of full-length and single-chain TCR variable (scTv). Yellow starbursts represent non-native cysteine residues in full-length yeast displayed TCR. **(B)** T cell receptor surface expression on $58^{-/-}$ T cells in the absence of CD8. Pertinent features of each display system as described. Subunits as indicated. δ=CD3 delta, ε=CD3 epsilon, γ=CD3 gamma, ζ= CD3zeta

CHAPTER TWO

IDENTIFICATION AND ENGINEERING OF HUMAN VARIABLE REGIONS THAT ALLOW EXPRESSION OF STABLE SINGLE-CHAIN T CELL RECEPTORS

Introduction

T cell receptors (TCRs) mediate recognition of a foreign or self-peptide presented in the context of a self major histocompatability (MHC) complex protein (59). During development, T cells that express the highest affinity receptors are deleted in the thymus (negative selection). Survival of the T cell, however, depends on a minimal affinity for self MHC (positive selection), assuring that T cell recognition is MHC restricted (76). After development, if recognition of peptide:MHC (pepMHC) is of sufficient affinity, the T cell becomes activated and secretes cytokines or causes lysis of the target cell. The affinity of TCR:pepMHC interactions are relatively low, with K_D values in the range of 1 to 500 μM (84, 100, 156).

In recent years, *in vitro* engineering by yeast and phage display has yielded T cell receptors with >1,000 fold improvements in affinity for class I MHC ligands (101, 148-150, 157, 158) and class II MHC ligands (154). To date there have been only a few MHC-restricted human TCRs engineered for improved affinity by phage display (reviewed in (159)). These receptors were engineered in a full-length TCR format (148-150), with the addition of two non-native cysteines in each of the TCR constant domains to facilitate TCR heterodimer formation through an interchain disulfide bond (160). The addition of this disulfide bond allows the TCR to be expressed on the surface of yeast (86) or phage (160).

Other affinity engineering efforts have focused on using a single-chain TCR (scTv) format, consisting of the variable domains of the T cell receptor connected by a flexible linker (Vα-linker-Vβ or Vβ-linker-Vα; called scTv here to distinguish it from antibody scFv fragments, and from single-chain TCRs that contain three domains, typically Vα, Vβ, and Cβ; (161-163)). Although analogous to antibody

fragments in many aspects, unlike antibody scFv, the scTv requires mutations to allow for stable expression of the variable domains in the absence of the constant domains (86). Previously, scTv fragments have been isolated by yeast display through random mutagenesis and subsequent selection with clonotypic antibodies to select for mutations that stabilize the scTv and allow for yeast surface expression and/or thermal stability (154, 164, 165). These stable scTv scaffolds were subsequently used for engineering receptors of enhanced affinity and for expression as stable, soluble scTv (154, 157, 166).

Like scFv fragments compared to the full antibody molecule, scTv fragments potentially afford significant advantages compared to the full-length TCR format for engineering, soluble protein expression, and therapeutic potential. Higher surface levels of the scTv can be achieved relative to the full-length TCR in the yeast display system (86). This allows for more TCR molecules to be displayed on the surface of each yeast cell, and improved avidity effects in the detection of peptide-MHC multimers. In addition, the scTv is produced as a single-polypeptide, which avoids the requirement for production of each TCR chain as separate polypeptides. This allows for production of large quantities of scTv fragments in relatively short time in comparison to proteins expressed and refolded in the full-length format. Most importantly, the scTv has potential applications as therapeutic or diagnostic reagents *in vivo* similar to scFv fragments, for example as bispecific single-chain molecules (167) or as immunotoxins (168). The scTv has the added potential of improved tissue penetration because of the reduced size of the scTv relative to the full-length receptor. This is especially important in cases where the TCR is directed against a tumor antigen, where tumor stroma could be targeted to effectively destroy cancerous cells (139, 140, 169).

Previous efforts to engineer single-chain TCRs were dependent on having probes available to assess folding of the TCR Vα and Vβ domains. In the well-characterized 2C (164) and 3L.2 (154) TCR systems, stabilized scTv fragments were identified by probing with clonotypic antibodies that recognized the conformation of the properly folded Vα and Vβ domains. Because the wild-type affinity of these receptors for specific pepMHC is relatively low, pep:MHC tetramers could not be

used to detect properly folded, stabilized scTv fragments. It has been known for some time that expression of the extracellular regions of the TCR as soluble protein is often problematic (59), and that the scTv format is typically unstable (86). The explanations for scTv instability are probably analogous to the long-standing observations that particular V_H families are more stable as scFv than other V_H families. Thus, the human V_{H3} family confers enhanced stability, but stability is influenced by framework residues and complementarity determining regions (CDRs) (170). Efforts to understand the structural basis of these effects are important because various scFv fragments as bispecific proteins are currently in various phases of clinical testing (171), and the development of a single, highly stable human scFv platform for different antigen specificities would be desirable (172). To this end, scFv libraries with randomized residues in all six CDR loops have been produced to obtain antibodies with high-affinity for various antigens (173).

In the current study, I explored whether human scTv fragments might also be developed using a single platform, allowing the natural antigens of the TCR, peptides bound to HLA, to be targeted. I used *in vitro* engineered, high-affinity T cell receptors in a single-chain format to isolate the first human stabilized scTv fragments (Vβ-linker-Vα), and I determined various features of the scTv that allowed them to be expressed as stable proteins, both on the surface of yeast and in soluble form from *E. coli*. High-affinity TCRs were used as templates for random mutagenesis (error-prone PCR), and stabilized scTv fragments were isolated by selection with soluble peptide:HLA-A2 tetramers. I describe the isolation of variants of two stabilized, human scTCRs, the A6 scTv that is specific for a peptide derived from the human T cell lymphotrophic virus Tax protein (peptide: $Tax_{11\text{-}19}$, LLFGYPVYV), and the 868 scTv that is specific for a peptide derived from the human immunodeficiency virus Gag protein (peptide: $SL9_{77\text{-}85}$, SLYNTVATL). Both of these TCRs used the same exceptionally stable Vα domain: Vα2 (IMGT: TRAV12 family), whereas other human scTv fragments that were not able to be displayed, contained other Vα regions. I also demonstrated that a single polymorphic residue at position 49 of the Vα2 controlled susceptibility to thermal denaturation and influenced the stability of the corresponding Vβ domain of the scTv. Furthermore, the two scTv fragments

could be expressed in soluble form from *E. coli* and were capable of recognizing their cognate peptides bound to HLA-A2 on antigen presenting cells.

Materials and methods

Antibodies, Peptide/HLA.A2 Tetramers, and Flow Cytometry

Antibodies used to detect yeast surface expression included: anti-human Vβ5.2, clone 1C1 (FisherThermo Scientific), anti-HA eptiope tag clone HA.11 (Covance), anti-human Cβ clone 8A3 (Endogen/Pierce), anti-human Cα clone 3A8 (Endogen/Pierce), goat anti-mouse IgG F(ab')$_2$ AlexaFluor 488 secondary antibody (Invitrogen), goat-anti-mouse IgG F(ab')$_2$ AlexaFluor 647 secondary antibody (Invitrogen), and streptavidin-phycoerythrin (SA:PE, BD Pharmingen). Peptides that bind to HLA.A2 (Tax$_{11\text{-}19}$: LLFGYPVYV, NYESO-Val$_{157\text{-}165}$: SLLMWITNV, SL9$_{77\text{-}85}$ (HIV-Gag): SLYNTVATL, and WT-1$_{126\text{-}134}$: RMFPNAPYL) were synthesized by standard F-moc (N-(9-fluorenyl)methoxycarbonyl) chemistry at the Macromolecular Core Facility at Penn State University College of Medicine (Hershey, PA.). HLA.A2 heavy-chain was produced as inclusion bodies and refolded *in vitro* with HLA.A2 binding peptides and human β-2 microglobulin as described (174). The HLA.A2 heavy chain contains a biotinylation substrate sequence which allows for *in vitro* biotinylation (Avidity, BirA enzyme) and subsequently for formation of strepavidin:phycoerythrin peptide/MHC tetramer. All tetramer and antibody staining of yeast cells was performed on ice for 45 minutes using ~1x10^6 cells. Cells were subsequently washed with 500 μL PBS/BSA (0.5%) and analyzed by flow cytometry with an Accuri C6 flow cytometer.

Cloning and Expression of Single-Chain and Full-Length T Cell Receptor Genes in Yeast Display Vectors

The single-chain genes for TCRs were synthesized by Genscript (Piscataway, NJ) with mutations originally isolated for improved affinity by phage display of full-length TCR with a non-native disulfide bond in the C regions (160). The TCRs are specific for various peptides in the context of HLA.A2: the A6 mutant clone 134 (K_D

= 2.5 nM) specific for Tax from human T cell lymphotrophic virus (148), the 1G4 mutant clone C49 (K_D = 1.3 nM) specific for NYESO (175), and an 868 mutant (K_D of at least 10 nM) specific for human immunodeficiency virus Gag peptide SL9 (149). For the 868 mutant, the most prevalent clone listed from CDR2β (YVRGEE, affinity mutations underlined) and CDR3α (CAVRGAHDYALN) was generated (149). The scTv genes (Figure 2.1), consisted of the variable domains attached by the linker region GSADDAKKDAAKKDGKS. The scTv genes were introduced into the yeast display vector pCT302 using NheI and XhoI restriction sites.

For construction of full-length TCR constructs, V region genes were introduced into human full-length VαCα and VβCβ as described (86, 160). In brief, Vβ genes of 868 and A6 were introduced into the p315 plasmid as a fusion to AGA-2 by ligation into NheI and BglII restriction sites and a BglII site was introduced into the Cβ gene. Vα genes were introduced into NheI and BamHI sites, and a BamHI site was introduced into the Cα gene, and both were cloned into a pCT302 secretion plasmid. The full-length TCR constructs contained a non-native Cys in each constant domain (α: Thr48Cys, β: Ser57Cys) to facilitate formation of a disulfide bond (160). C region cysteine residues that normally participate in an interchain disulfide bond in the stalk region were replaced with a stop codon and the Cβ cysteine at position 71 was mutated to a serine (Cβ: Cys71Ser).

scTv fragments and VβCβ for full-length TCR constructs were expressed from yeast display plasmid pCT302 (Vβ-L-Vα scTCRs) (176) or p315 (VβCβ, generously provided by K. Dane Wittrup, MIT) which contain a galactose-inducible AGA2 fusion and allow for growth in Trp- media or Leu- media respectively. VαCα for full-length TCR constructs was expressed from pCT302-secreted, a galactose-inducible system that allows for growth in Trp- media. Gene induction involved growth of transformed EBY100 yeast cells to stationary phase in selection media followed by transfer to galactose containing media.

Construction, Display, and Selection of Mutated scTv Yeast Display Libraries

Error-prone PCR was used to generate random mutations, as described previously (86). PCR products were electroporated along with NheI and XhoI digested vector into yeast strain EBY100 to generate libraries by homologous recombination. Random mutational libraries of the A6, 868, and 1G4 scTv genes were induced in galactose containing media (SG-CAA) for 48 hours, washed with 500 μL 0.5% PBS/BSA, and stained with corresponding pep:MHC SA/PE tetramers (100 nM). Cells were washed (5 ml, 0.5% PBS/BSA), and the most fluorescent cells were selected using either a MoFlo (Cytomation) or a FACS Aria (BD Bioscience) high-speed sorter. In some cases, libraries were subjected to additional rounds of thermal stability sorting whereby yeast cells were incubated at higher temperatures (A6: 42°C for 45 minutes, 868 40°C for 30 min) prior to staining. The percent collected and pertinent details of each sort are provided below.

A6 scTCR. 5 Sorts on initial error-prone Libray: Sort 1:, top 0.5%, 500,000 cells, Sort 2: top 0.5%, 210,000 cells, Sort 3: top 0.5%, 150,000 cells, Sort 4: top 0.5%, 100,000 cells, Sort 5: top 0.5%, 100,000 cells. After 5 sorts, a clone was isolated that showed detectable binding to peptide:MHC tetramer. This clone was then used as a template for an additional round of error-prone mutagenesis. The second error-prone library was sorted twice: Sort 2-1: top 0.5%, 300,000 cells, Sort 2-2: top 10%, 1,000,000 cells. Sort 2-3: after induction in galactose containing media, preincubated yeast at 42°C in water bath for 45 minutes and then stained with Tax/HLA.A2 tetramer at 100 nM for 45 minutes on ice and collected top 0.7%, 350,000 cells. Individual yeast cell clone were selected from the population isolated after this sort and tested for resistance to thermal denaturation at 40°C (Clones X1-X20).

868 scTCR. 1 Sort on initial error-prone library and 1 round of temperature stability sorting. Sort 1: top 2%, 500,000 cells, Sort 2: after induction in galactose containing media, preincubated yeast at 40°C in water bath for 30 minutes, stained with Tax/HLA.A2 tetramer at 100 nM for 45 minutes on ice, and collected the top 0.5%, 21,000 cells. Cells were expanded in selection media and individual yeast

clones were isolated and tested for resistance to thermal denaturation at 40°C (Clones Z1-Z20).

Cloning of Single-Site or Multiple-Site scTv Mutants

The $Phe_{V\alpha 49}Ser$ mutation was introduced using Quikchange Lightning Kit (Stratagene, Agilent Technologies) with forward primer: 5' GGTAAATCTCCA GAATTGATCATGTCCATCTACTCTAATGGTGACAAAGAAG 3' and reverse complement primer: 5' CTTCTTTGTCACCATTAGAGTAGAT GGACATGATCAATTCTGGAGATTTACC 3'. The 868 scTv with CDR2β (Sequence: YYEEEE) and CDR3α (Sequence: CAVRTNSGYALNFG) wild-type residues was synthesized by Genscript. The high affinity mutations for each CDR were introduced into this template by splicing by overlap extension (SOE) PCR (177).

Expression in E. coli, Folding, and Biotinylation of Soluble scTv Fragments

The 868-Z11 and A6-X15 scTv mutants were introduced into the pET28a expression vector using NcoI and EcorI (868-Z11, forward primer: 5'TATACCATGGGCAGCAGCCATCATCATCATCAT CACAGCAGCGGCCTGGTGCCGCGCGGCAGCGAAGCTGGTGTTACTCAATC TCC 3' 868-Z11 reverse primer: AAATGAATTCTTAAATATGTGGAGTAACCCAAAAAGAAG TACC). Plasmids were transformed into the BL21 cell line, and after culture and gene induction cells were passed through a microfluidizer and inclusion bodies were isolated and protein purified as described (178). scTv proteins were folded from inclusion bodies and purified with Ni agarose resin (Qiagen, Valencia, CA) followed by gel filtration (Superdex 200). Folded proteins were biotinylated using *N*-hydroxysuccinimide (NHS) biotin ester (EZ-Link Sulfo-NHS-LC-Biotin Kit, Pierce/Thermo Scientific). Biotinylation was verified by gel-shift with streptavidin by SDS-PAGE.

Binding of scTv Proteins Measured by Surface Plasmon Resonance

The binding of purified refolded scTv proteins to cognate peptide-HLA.A2 was monitored with surface plasmon resonance using a Biacore 3000 instrument. Complexes of the Tax_{11-19} and $SL9_{77-85}$ peptides with HLA.A2 were generated by refolding from bacterially-expressed heavy chain and β2m inclusion bodies as previously described (179) . Due to the high affinities of the scTv proteins, a kinetic titration assay was utilized, in which increasing concentrations of analyte were sequentially injected over the surface without the requirement for disruptive regeneration injections (180). Experiments were performed with 868-Z11 and A6-X15 amine coupled to a standard CM5 sensor chip. Peptide/MHC analyte was sequentially injected at various concentrations. The amount of immobilized scTv was kept below 500 RU and the flow rate was set to the maximum of 100μL/min to minimize mass transport effects. Data were analyzed using Biaevaluation 4.1 as described (180). Solution conditions were 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P-20, 25 ºC. SPR experiments were performec by Francis Insaidoo and Kurt Piepenbrink in Professor Brian Baker's Lab (U. Notre Dame).

Binding of scTv Proteins to Peptide-Loaded Antigen Presenting Cells

To examine binding of soluble scTv protein to peptide presented by HLA.A2 on cells, T2 cells ($HLA.A2^{+}$) were incubated for 2 hrs with various concentrations of peptides. Cells were then washed twice with 0.5% PBS/BSA, and incubated at room temperature for 30 minutes with biotinylated scTv at various concentration. Cells were washed twice with 0.5% PBS/BSA, followed by incubation with SA:PE for 30 minutes at room temperature. Cells were washed twice and analyzed using an Accuri C6 Flow Cytometer.

Results

Yeast Display of Human Single-Chain TCRs

To determine whether human TCRs could be expressed in single-chain formats, I cloned three human TCRs (Figure 2.1A) that had high-affinity for their respective pep-HLA.A2 ligands, thus allowing the pepMHC to be used as high-affinity probes for properly folded and associated VαVβ binding sites. The TCRs were engineered previously for higher affinity, using a full-length TCR format and phage display (148, 149, 175). The variable domains contained various mutations in the CDRs that conferred high-affinity, and they were fused by a linker peptide (L) in the orientation Vβ-L-Vα (Figure 2.2). The single-chain constructs were cloned at the C-terminus of the yeast cell wall protein Aga2, followed by a hemagglutinin (HA) peptide tag for detection of displayed proteins.

Yeast cells containing the 1G4, A6 and 868 scTv fusions were induced, and the cells were stained with an anti-HA antibody (Figure 2.1B). All three constructs showed high levels of expression of the HA tag, as evidenced by a positive population (the negative population has been found in all yeast displayed systems, and is due to a fraction of yeast cells that lose the plasmid during induction). To detect properly folded VαVβ domains, the respective peptide/A2 tetramers were used for staining the same induced yeast cells. As expected, based on previous studies with various scTv fragments (86), neither the 1G4 nor A6 scTv were positive with their respective cognate pep/HLA.A2 ligands (Figure 2.1B), consistent with the absence of properly folded scTv proteins on the surface of yeast. Staining of the 1G4 and A6 cells with an antibody to Vβ13 was also negative (unpublished data), confirming that these scTv proteins were not expressed on the yeast surface. Surprisingly, the 868 scTv was positive for staining with its cognate ligand, SL9/HLA.A2, even at the concentration of 10 nM (Figure 2.1B). This represented the first time that a scTv fragment could be detected as a properly folded protein, without the need for random mutation and selection (86, 154, 164).

Engineering of Stabilized Surface Displayed scTv Fragments by Random Mutagenesis

Although the features of the 868 V regions that allowed display were unknown, this TCR uses a Vβ (Vβ5) that differs from 1G4 (Vβ13) and A6 (Vβ13), while it uses a Vα that is similar to A6 (Vα2). This may imply that the Vβ5 region of 868 confers stability on the protein. To gain insight into the mechanisms that enable scTv stability and surface display, I performed mutagenesis and selections for displayed proteins. First, to determine if the 1G4 and A6 scTv could be stabilized by specific V region mutations, the scTv genes were subjected to rounds of random mutagenesis and libraries were sorted with pep/HLA.A2 tetramers to isolate scTv mutants with improved surface expression. Despite multiple efforts, through various rounds of mutagenesis and sorting with NYESO/HLA.A2 tetramers, yeast displayed 1G4 scTv could not be isolated (data not shown).

In contrast, stabilized mutants of the A6 scTv were isolated after mutagenesis and sorting with the Tax/HLA.A2 tetramers (100 nM). Previous experiments with the mouse TCRs 2C and 3.L2 demonstrated that selection of scTv fragments with increased resistance to thermal denaturation yielded mutants with significantly higher levels of display that correlated with soluble secretion efficiency (154, 165). Consequently, the A6 scTv library was sorted with Tax/HLA.A2 tetramer after incubation at a higher temperature (42°C) and several mutants, including a clone called A6-X15 with improved yeast surface levels were isolated (Figure 2.3). The scTv mutant A6-X15 (fluorescence intensity FI=1600) was expressed at higher surface levels than the A6 template scTv (FI=50) or the full-length TCR (FI=200) (Figure 2.3), with stabilized C region cysteines, as monitored by Tax/HLA.A2 tetramer. In the full-length A6 TCR, each of the Cα and Cβ antibodies detected higher surface levels than tetramer, suggesting that the fully folded and assembled dimer represents only a subset of the total α and β chains on the surface. Notably, introduction of the Vβ mutations from A6-X15 into the 1G4 scTv, which shares Vβ usage with A6, did not allow for surface expression of the 1G4 TCR (data not shown).

Based on our previous results (154, 165), I also sought to identify mutants of the 868 scTv that would exhibit increased stability. The 868 scTv gene was randomly mutagenized by error-prone PCR, and the library was subjected to several rounds of sorting with SL9/HLA.A2 tetramer, including a round after incubation at 40°C. Various temperature stable clones were isolated, including a mutant called 868-Z11 that bound to SL9/HLA.A2 tetramer at concentrations as low as 1 nM as detected by flow cytometry (Figure 2.4 and data not shown). Clone 868-Z11 exhibited improved surface levels that were almost ten-fold improved relative to the 868 scTv template (Figure 2.4). In addition, like the A6 TCR, the scTv forms of the 868 TCR were also detected at higher levels than the full-length format that contained C regions with the stabilizing cysteines (Figure 2.4). This demonstrated that the single-chain format allows for much higher surface levels of productively arranged Vα:Vβ pairs relative to the full-length display format. This is consistent with a previous finding using the mouse 2C system, comparing the temperature-stabilized scTv and full-length TCR formats (86).

To examine what mutations in the A6 and 868 scTv conferred enhanced yeast surface levels and temperature stability, various clones, including A6-X15 and 868-Z11, were sequenced (Figure 2.5). The A6-X15 clone showed only mutations in the Vβ13 region, with no mutations in the Vα2. Several of these Vβ mutations (A52V, N63D, N66K, Q106L) were also shared with the stabilized clone A6-X3 (Figure 2.4A). Thus, it appeared that in A6, the Vα2 region, but not the Vβ13 region, was sufficiently stable to allow its expression, proper folding, and assembly with the mutated Vβ on the yeast surface.

In contrast to the A6 scTv mutants, the stabilized 868 scTv clones did not show any shared mutations in the Vβ region, but they contained a single shared mutation in the Vα2 region, F49αS (Figure 4B) (Note that the numbering follows that used in the pdb file of A6; based on IMGT sequences, this residue is numbered 55). Interestingly, the Ser_{49} residue is also found in the Vα2 of the A6 TCR, and represents a polymorphism at this position (Figure 2.2).

Polymorphic Residue 49 of Vα2 Influences Vα and Vβ Domain Stability

As indicated, the sequences of the five most stable 868 scTv clones (out of 20) isolated after temperature stability sorting shared a single Vα mutation, $Phe_{V\alpha 49}Ser$, and this was the only Vα mutation present in the most stable 868 clone, 868-Z11 (Figure 2.5B). To assess whether this mutation alone, and not the Vβ mutations, was responsible for the enhanced stability of the 868-Z11 mutant, the $Phe_{V\alpha 49}Ser$ mutation was introduced into the 868 scTv. Staining of this mutant after incubation at 40°C showed that this single mutation provided the protein with improved resistance to thermal denaturation, resulting in over a ten-fold increase in surface level (Figure 2.6).

Relative Effects of CDR and $Ser_{\alpha 49}$ *Mutations on Yeast Display of scTv*

Our previous work with the mouse TCRs 2C and 3.L2 have shown that some CDR mutations can also confer increased stability to the scTv format (154, 164). It is possible that the 868 CDR mutations that were selected for higher affinity binding to SL9/HLA.A2 also contributed to stability of the scTv format. Accordingly, the wild-type CDR residues were cloned into the 868 scTv format (WT), with either a $Phe_{\alpha 49}$ or $Ser_{\alpha 49}$ residue. To approach this question, I could not use the tetramer for detection, due to the low affinity of the wild-type TCR. Thus, an antibody to the TCR Vβ5 domain was used as a surrogate to assess surface levels (Figure 2.7). The WT 868 scTv with the $Ser_{\alpha 49}$ residue showed significantly improved surface levels, relative to the WT 868 scTv with the $Phe_{\alpha 49}$ residue, which was barely detectable with the anti-Vβ5 antibody. Although the $Ser_{\alpha 49}$ residue yielded enhanced surface levels with the high-affinity mutations (MUT) in the CDRs, in this case even the scTv with the $Phe_{\alpha 49}$ residue was easily detected with the anti-Vβ5 antibody (Figure 2.7). Thus, the CDR mutations that yielded higher affinity also contributed to enhanced stability. I have observed a reciprocal selection scenario, in which stabilizing mutations in the CDR1α of a scTv also yielded higher affinity (154).

To further investigate whether the high-affinity mutations in the CDR2β and/or the CDR3α were responsible for the enhanced surface stability, the wild-type

CDR2β and CDR3α sequences were individually introduced into the template high-affinity 868 scTv, with the $Phe_{\alpha 49}$ or $Ser_{\alpha 49}$ residue, and yeast surface levels were monitored by staining with anti-Vβ5 antibody. The high-affinity mutations in CDR3α conferred improved Vβ surface levels as monitored with the anti-Vβ5 antibody, in comparison to the WT scTv with $Phe_{\alpha 49}$ (Figure 2.7). In contrast, the high-affinity mutations in CDR2β did not increase substantially the Vβ surface levels in the presence of $Phe_{\alpha 49}$ but appeared to act synergistically in the presence of $Ser_{\alpha 49}$. Thus, it appears that both CDR mutations and the $Ser_{\alpha 49}$ mutation provided enhanced stability to the scTv format of this TCR. The maximal scTv surface levels observed for binding of SL9/HLA.A2 tetramers to proteins that contained the high-affinity mutations (Figure 2.7) also substantiated these findings.

Expression of Soluble Human scTv Proteins in E. coli, and Peptide:HLA.A2 Binding

Our previous studies with mouse TCRs 2C and 3.L2 have shown that the generation of yeast surface displayed mutants of the scTv proteins allowed the mutants to be expressed in *E. coli* in both secreted form and refolded from inclusion bodies (154, 181, 182). To determine if this were possible with the human scTv mutants, the A6-X15 and 868-Z11 scTv genes were cloned into an *E. coli* expression vector and the induced *E. coli* cells expressed the expected size proteins (data not shown). The scTv proteins were refolded from inclusion bodies with yields of greater than 1 mg/L of culture, yielding purified scTv proteins of the expected 30 kDa (Figure 2.8A).

Two different binding studies were conducted. First, surface plasmon resonance (SPR) was performed with purified peptide/HLA.A2 binding immobilized scTv to determine solution binding affinities and kinetics. A kinetic titration experiment was performed, obviating the need for potentially destabilizing regeneration steps necessary when high affinity interactions are studied with SPR (180). Analysis of the data for the A6-X15-Tax/HLA.A2 and 868-Z11-SL9/HLA.A2 interactions yielded nM and pM binding affinities for the A6-X15 and 868-Z11 interactions, respectively (Figure 2.8B-C). The values determined reflected the affinities seen with the full-length proteins and confirmed the single-chain constructs

retained specificity and high-affinity for their cognate ligand. Differences with the previously published affinities (148, 149, 183) may reflect difficulties quantifying high affinity binding via SPR, or possibly an influence of the mutations generated via yeast display, or the linker, on binding affinity. These possibilities are currently being investigated.

In a second approach, I determined whether cell surface pep/HLA.A2 complexes could be detected with soluble scTv protein. The 868 scTv protein was biotinylated using a biotin-succinimidyl cross-linking agent that reacts with free amines (the 868 scTCR linker contains multiple lysines, whereas the scTv contains no lysines in the CDR loops). The HLA.A2-positive human cell line T2 was loaded with SL9 peptide or Tax (868-null) peptide at 10 μM and cells were subsequently incubated with various concentrations of biotinylated-868-Z11 scTv followed by streptavidin:PE at room temperature (Figure 2.9A). Soluble 868-Z11 scTv detected SL9 peptide loaded cells at scTv concentrations below one nanomolar, whereas cells loaded with the null peptide Tax were not positive even at the highest concentration of 868-Z11 scTv (Figure 2.9A). The concentration that yielded 50% maximal staining, an estimate of the K_D of the reaction, was approximately 4 nM scTv (Figure 2.9B).

To examine the peptide sensitivity of binding by the scTv, the SL9 peptide was titrated on T2 cells and the antigen presenting cells were stained with biotinylated 868-Z11 scTv at 100 nM (Figure 2.9C). Without any optimization of staining signal, the scTv detected the peptide at concentrations as low as 10 nM. The scTv fragments appear to be quite stable, as even after storage at 4°C for over one year, the protein was able to bind target cells loaded with low nanomolar peptide concentrations (data not shown).

Discussion

It has been known for many years that the expression and crystallization of the extracellular region of αβ TCRs have posed significant problems, when compared to either antibodies or MHC proteins (59). The explanation for this difficulty lies, at

least in part, in the behavior of TCR variable regions. This also accounts for problems that have plagued efforts to use single-chain TCR V-region fragments in the same way that has been done with antibody single-chain Fv fragments (86). Here, I show that it is possible to at least in part overcome these inherent problems by using a specific V region family, Vα2 (IMGT: TRAV12 family). This Vα region appears to be over-represented among human T cells directed against a number of HLA.A2 associated antigenic peptides, perhaps most notably peptides from MART1/MelanA (ELAGIGILTV) and Epstein Barr Virus protein BMLF1 (GLCTLVAML) (184-186). It is possible that the inherent stability of Vα2, observed in the context of scTv proteins described here, might contribute to its preferential use in some T cell responses, and in the original isolation of the HTLV-1 Tax and HIV-1 Gag-specific TCRs, A6 and 868 (149, 187).

In previous studies, I have attempted to provide a possible molecular rationale for V region mutations that enabled the yeast surface display of mouse scTv (86, 154, 164, 165) and individual Vβ regions (188). The underlying premise was based on our observations that surface display was directly correlated with thermal stability of the scTv (154, 166, 189). In some cases, a possible explanation involved enhanced hydrophilicity at positions where a residue was buried at the V:C interface, or mutations at the Vα:Vβ interface that improved domain association. However, a significant number of mutations, such as those present within or at the base of CDR loops, have less obvious explanations. For example, it is unknown how the CDR3α or CDR2β mutations in the 868 scTv that conferred high-affinity also yielded enhanced surface display and stability, in the unbound state. In addition, A6 scTv mutants with improved stability each contained a mutation within CDR2β, $Ala_{V\beta52}Val$. We have argued that some of the CDR mutations could confer local stability on the CDR loops, based on modeling predictions (101). Similar results with stabilizing CDR mutations in scFv fragments have also been observed (170, 172).

Additional mutations in the framework 3 region or in hypervariable 4 region of the Vβ domains may also have contributed to the stability of the A6 and 868 scTv fragments. In the case of A6, the $Asn_{V\beta62}Asp$ and $Asn_{V\beta66}Lys$ mutations within framework 3 were conserved among isolated clones. Although the contributions of

each individual mutation has not been determined, their conservation among 14 out of 20 scTv clones of the A6 TCR (data not shown) suggest that they play a role in surface display and stability.

The mechanism by which residue 49 of the Vα domain confers stability is also not clear. It is near the CDR3β (e.g. it is 2.8Å from Glu105β) loop, such that it is possible its primary role is in stabilization of the Vα:Vβ interface (Figure 2.10), a location that has been proposed should be a priority in the design of stabilized scFv fragments (172). This area of the Vβ (i.e. near Glu105β, which can be encoded by either N-region addition, the D gene, or the N-terminus of the Jβ gene) could in turn influence the stabilizing potential of the Vα and Ser49α. The residue adjacent to Glu105β, Gln106β, was mutated to Leu in each of the surface displayed scTv of A6, suggesting that this region can be further optimized for enhanced stability in the context of the Ser49α residue. Furthermore, CDR3α and CDR3β stabilizing mutations (L104αP and T105βA) have been identified previously in the mouse 2C scTv (164). Thus, it is apparent that various CDR and framework regions can have complex and interdependent impacts on the biophysical properties of the scTv molecules, not unlike antibody scFv fragments.

Studies with soluble full-length TCRs (VαCα/VβCβ) have shown that an interchain disulfide bond formed by a pair of introduced cysteines in the Cα and Cβ regions can confer additional stability on the molecule, enabling expression as a soluble protein (160). This form of the TCR has been used for binding studies, and as a platform for affinity engineering (148-150). The design of stable scTv fragments that bind to the specific pepMHC ligands, as shown here, provides additional opportunities to use the scTv as a platform for various applications as has now been widely done with antibody scFv fragments (190). These include the use of display technologies for affinity engineering (176, 191) and more facile production of biophysically well-behaved single-chain proteins in *E. coli* (192, 193). The scTv form also provides a platform for use in chimeric, single-chain antigen receptors (155) or in soluble form as bispecific fusions that could include, anti-CD3 antibodies, toxins, or cytokines (167, 172).

Based on the diverse sequences of the HLA.A2-restricted peptides that have elicited V$\alpha 2^+$ T cells (e.g. MART1, ELAGIGILTV; EBV, GLCTLVAML; Tax, LLFGYPVYV; Gag, SLYNTVATL) I anticipate that it should be possible to generate V$\alpha 2^+$ scTv proteins against a wide array of diverse antigens. Two structures are available for V$\alpha 2^+$ TCRs in complex with pep-HLA.2, the A6 TCR:Tax/A2 complex (194) and the MEL5 TCR/MART-1/A2 complex (195). The two TCRs dock in almost identical orientations on their pep-A2 ligands, and they share CDR1α interactions with both the $\alpha 2$ helix and peptide positions 1 and 4. Peptide specificity appears to be achieved in large part through interactions with CDR3β, providing support to the notion that the scTv proteins described here, which use distinct Vβ regions, can maintain their antigen specificity through the involvement of other CDRs.

Figures

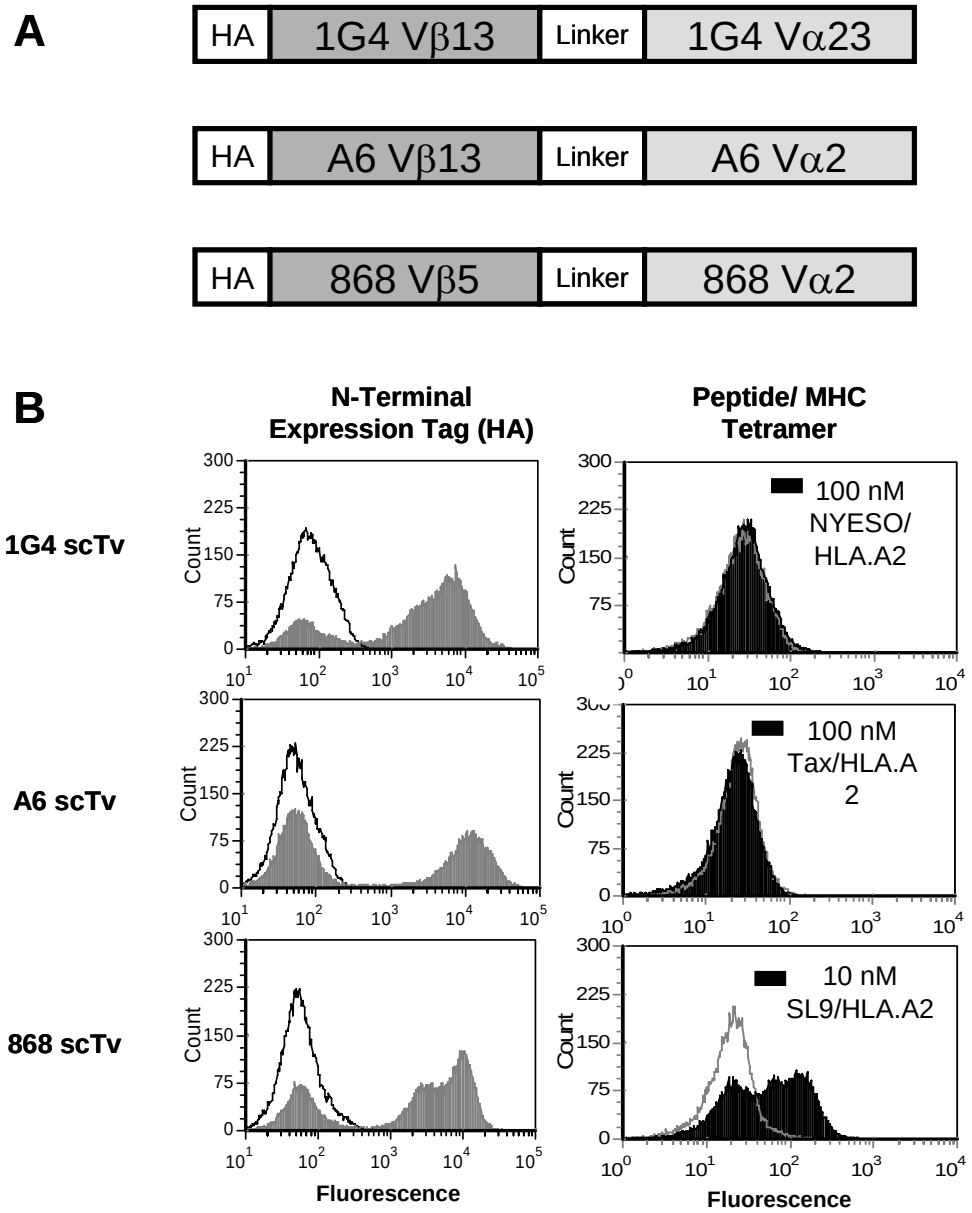

Figure 2.1 Yeast Surface Display of Single-Chain TCR Variable Fragments (scTv). (A) Schematic of scTv fusions for the human TCRs 1G4, A6, and 868, which recognize peptides NYESO, Tax, and HIV-Gag (SL9) in the context of HLA.A2. (148-150) **(B)** Yeast surface expression of scTv fusions was monitored for expression of N-terminal expression tag (hemagluttanin (HA), solid gray) with anti-HA antibody and goat anti-mouse IgG alexa 488 secondary antibody, or secondary antibody only as a control (black line). The negative peak in each histogram is due to yeast that have lost plasmid, and serves as an internal control for each induced yeast sample. To assess display of properly folded scTv fusions, cells were incubated with pep:HLA.A2 streptavidin:phycoerythrin tetramers. Null tetramer staining histograms are shown (gray line) as a control, along with the cognate tetramers, as indicated (black, solid).

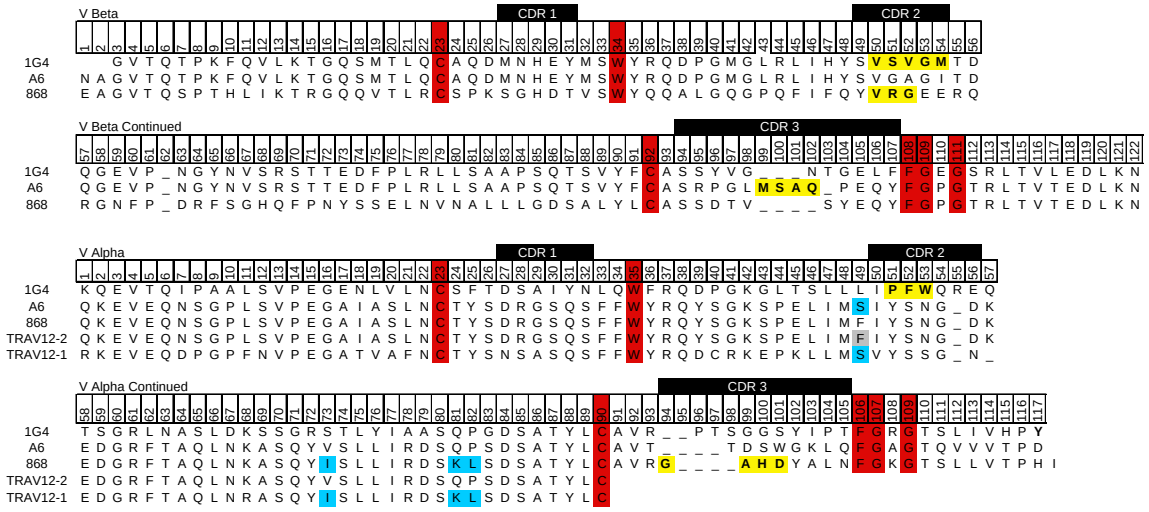

Figure 2.2. Sequences of scTv Templates. Yellow: high-affinity CDR mutations as identified for 1G4, A6, and 868 (148-150). Orange: conserved immunoglobulin fold residues. Both A6 and 868 belong to subfamily TRAV12-2, but 868 shares some residues with TRAV12-1 (blue). Position Vα49 is either a polymorphic residue or a subfamily variable residue.

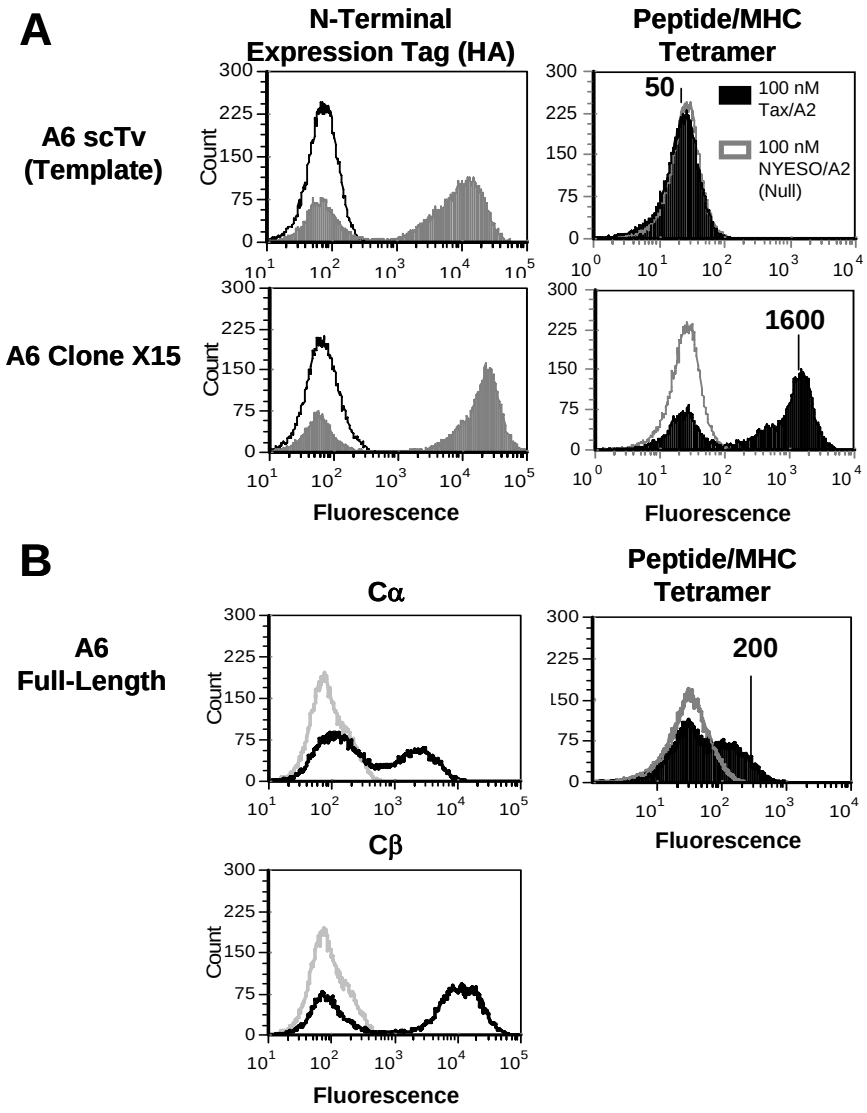

Figure 2.3. Isolation of Stabilized A6 scTv Mutants and Yeast Display of With Full-Length A6 TCR. (A) The high-affinity A6 TCR was cloned as a scTv , and a library of random mutants was sorted with Tax/HLA.A2 tetramer. Clone A6-X15, isolated after two rounds of mutagenesis and selection with Tax/A2 tetramer, was stained with anti-HA antibody (left panels, solid gray histogram), secondary antibody only (left panels, black line histograms), 100 nM Tax/A2 tetramer (right panels, solid black histogram), or 100 nM null peptide NYESO/A2 tetramer (right panels, gray line histogram). **(B)** The full-length A6 TCR with β chain expressed as a fusion to AGA-2 and α chain secreted was used to compare full-length and single-chain formats. Surface expression of α and β chains were monitored with anti-Cα or anti-Cβ antibody (left panels, black line histograms) or with goat anti-mouse IgG secondary antibody only as a control (left panels, gray line histograms). Cells were stained with 100 nM Tax/A2 tetramer (right panel, solid black histogram), or 100 nM null peptide NYESO/A2 tetramer (right panel, gray line histogram). Mean fluorescence units of positive peaks are shown.

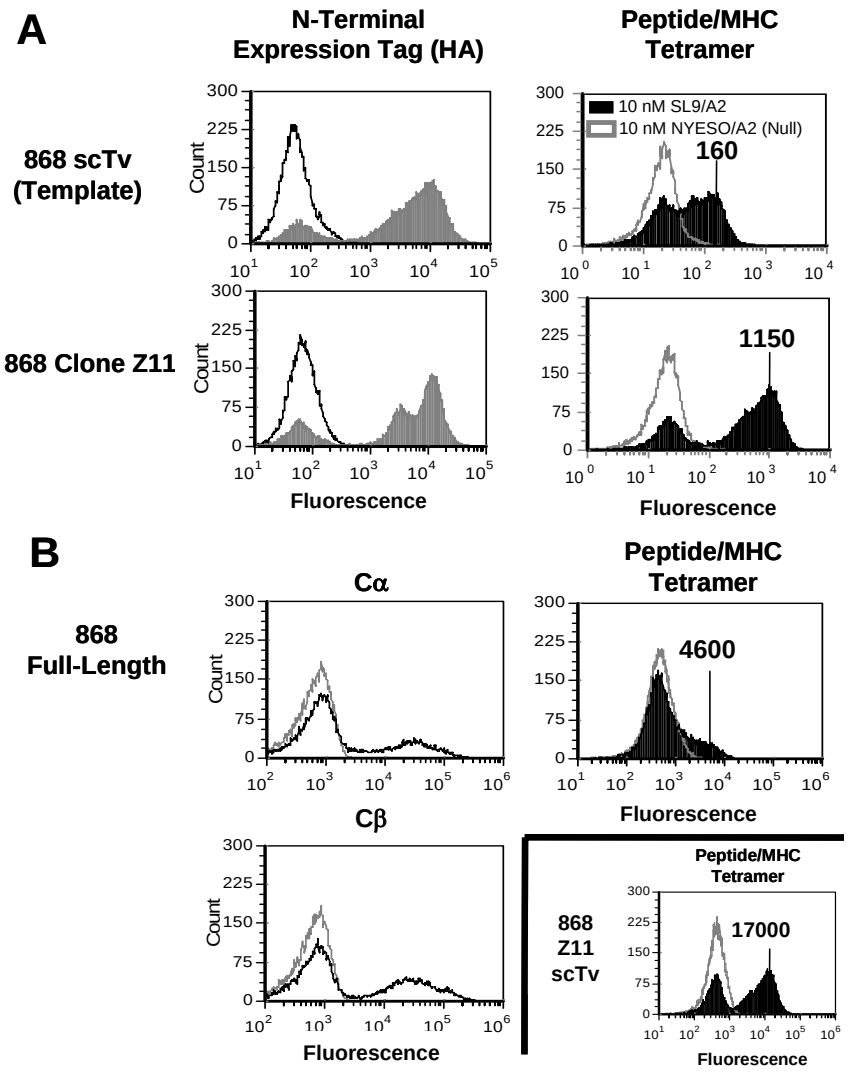

Figure 2.4. Isolation of Stabilized 868 scTv Mutants and Yeast Display With Full-Length 868 TCR. (A) The high-affinity 868 TCR was cloned as a scTv, and a library of random mutants was sorted with HIV Gag SL9/HLA.A2 (SL9/A2). Clone 868-Z11, isolated after one round of mutagenesis and selection with SL9/A2 tetramer, was stained with anti-HA antibody (left panels, solid gray histogram), secondary antibody only (left panels, black line histograms), 10 nM SL9/A2 tetramer (right panels, solid black histogram), or 10 nM null peptide NYESO/A2 tetramer (right panels, gray line histogram). **(B)** The full-length 868 TCR with β chain expressed as a fusion to AGA-2 and α chain secreted was used to compare full-length and single-chain formats. Surface expression of α and β chains were monitored with anti-Cα or anti-Cβ antibody (left panels, black line histograms) or with goat anti-mouse IgG secondary antibody only as a control (left panels, gray line histograms). Cells were stained with 10 nM SL9/A2 tetramer (right panel, solid black histogram), or 10 nM null peptide NYESO/A2 tetramer (right panel, gray line histogram). Note that the 868-Z11 scTv staining with tetramer is shown again, as in this experiment different flow cytometry settings were used compared to (A), yielding the indicated mean fluorescence units for comparison with full-length 868 TCR.

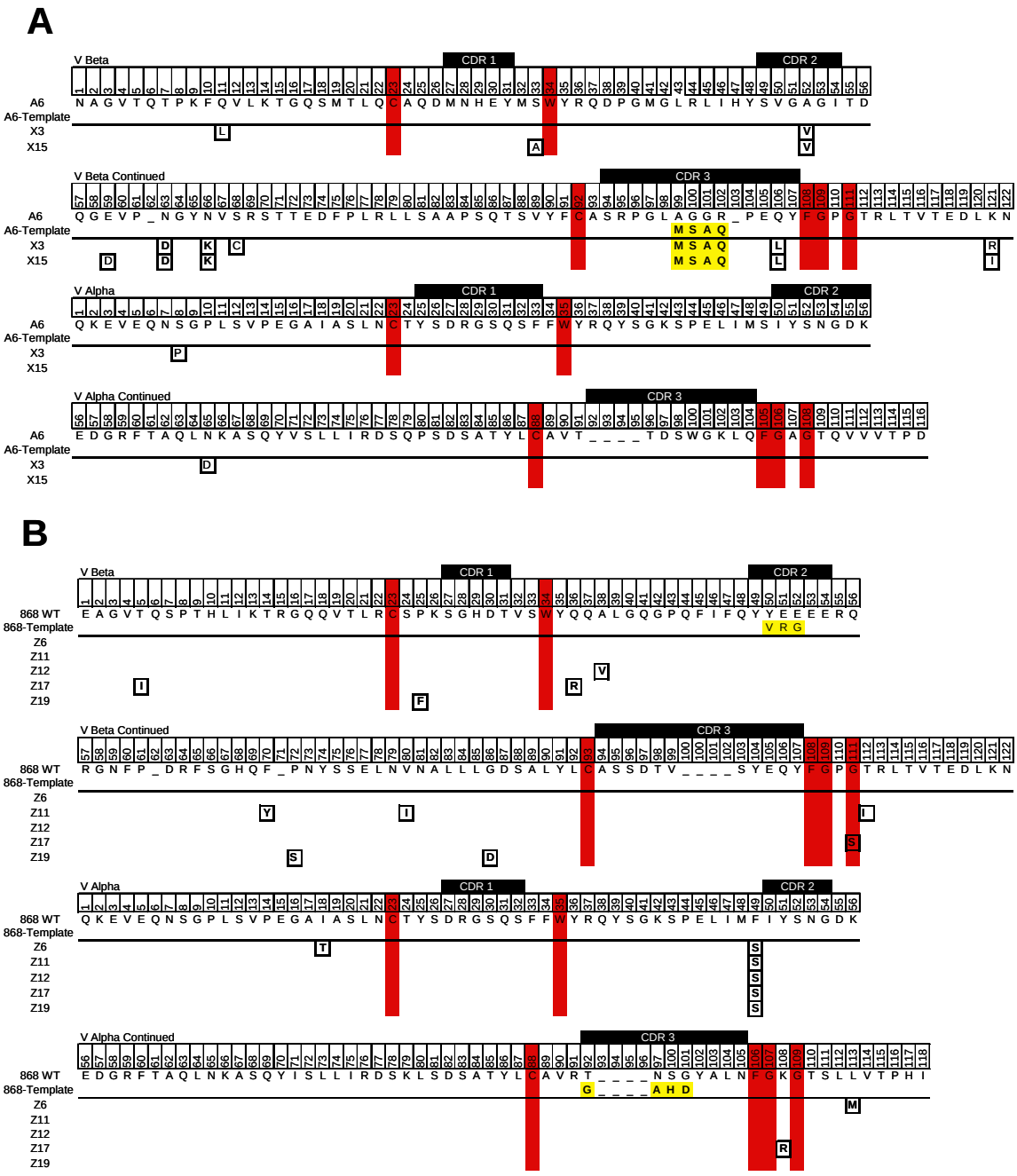

Figure 2.5. Sequences of Selected A6 and 868 scTv Mutants Showing Highest Surface Levels Following Thermal Denaturation. (A) Mutants of the A6 scTv or the **(B)** 868 scTv were isolated after mutagenesis of the scTv genes, followed by surface expression, high-temperature exposure, and selection with tetramers. Sequences are coded as follows: yellow, high-affinity CDR mutations for A6 (148) and for 868 (149); orange, conserved immunoglobulin fold residues, black-boxed residues, mutations identified in selected mutants. Note: for ease of comparing with published structures, residues of each variable domain of the scTv are numbered sequentially, and thus do not correspond to IMGT nomenclature.

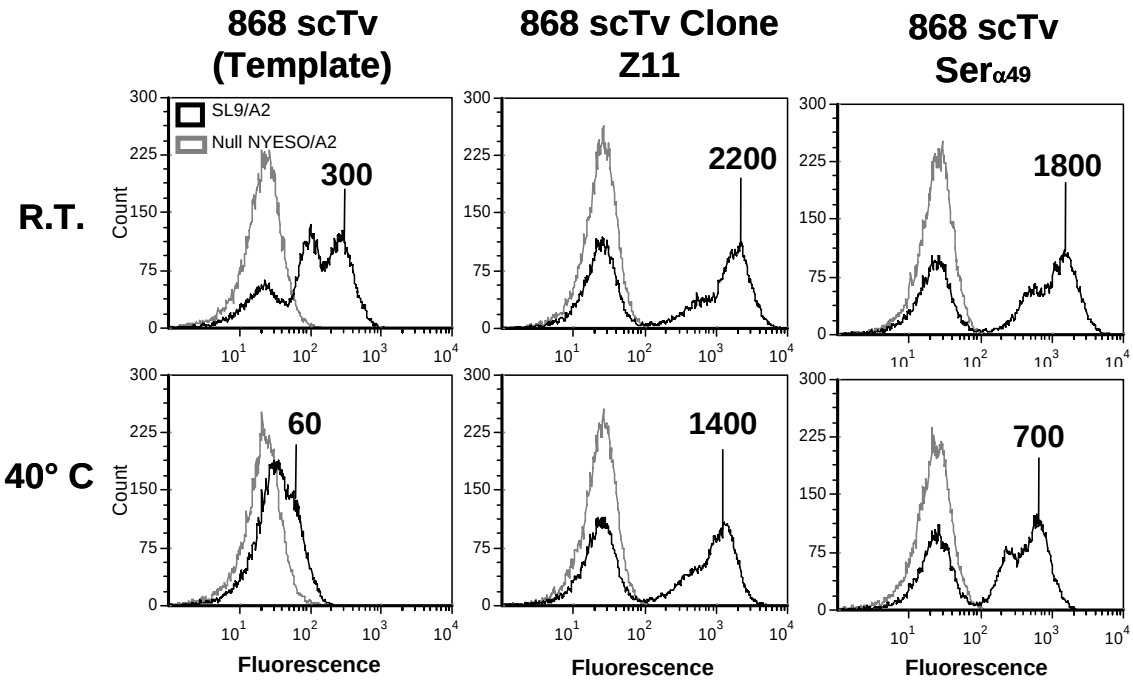

Figure 2.6. Polymorphic Residue 49 of the Vα2 Region Influences Thermal Stability of the 868 scTv. The serine polymorphism at position 49 was introduced into the 868 scTv template. Yeast cells were incubated at room temperature (top row) or at 40°C for 30 min and then stained with 10 nM SL9/A2 SA:PE tetramer (black line histograms) or null peptide Tax/A2 SA:PE tetramer (gray line histograms). Mean fluorescence units of positive peaks are shown.

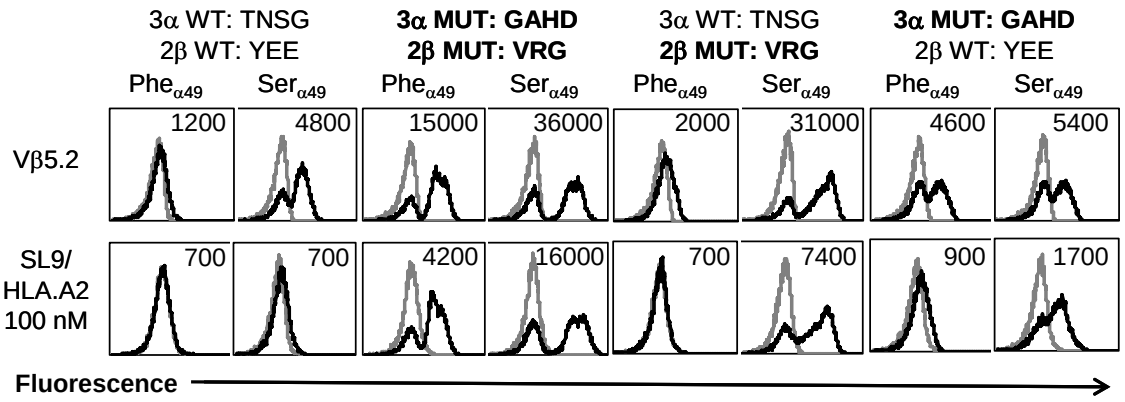

Figure 2.7. CDR Mutations and the Phe$_{Vα49}$Ser Mutation Each Improve the Surface Display of the 868 scTv. Various 868 scTv proteins were expressed in the yeast display system with wild-type CDR residues or with high-affinity CDR mutations (see Figure 2.5B), with either Phe or Ser at position α49. Top row, cells stained with anti-Vβ5.2 antibody and goat anti-mouse (black line histograms) or secondary antibody only (gray lined histograms). Bottom row, surface expression was also monitored with SL9/A2 tetramers (black line histograms) or null peptide Tax/A2 tetramers (gray lined histograms). Mean fluorescence units of positive peaks are shown (or in cases where only a negative peak is present, the mean of this peak).

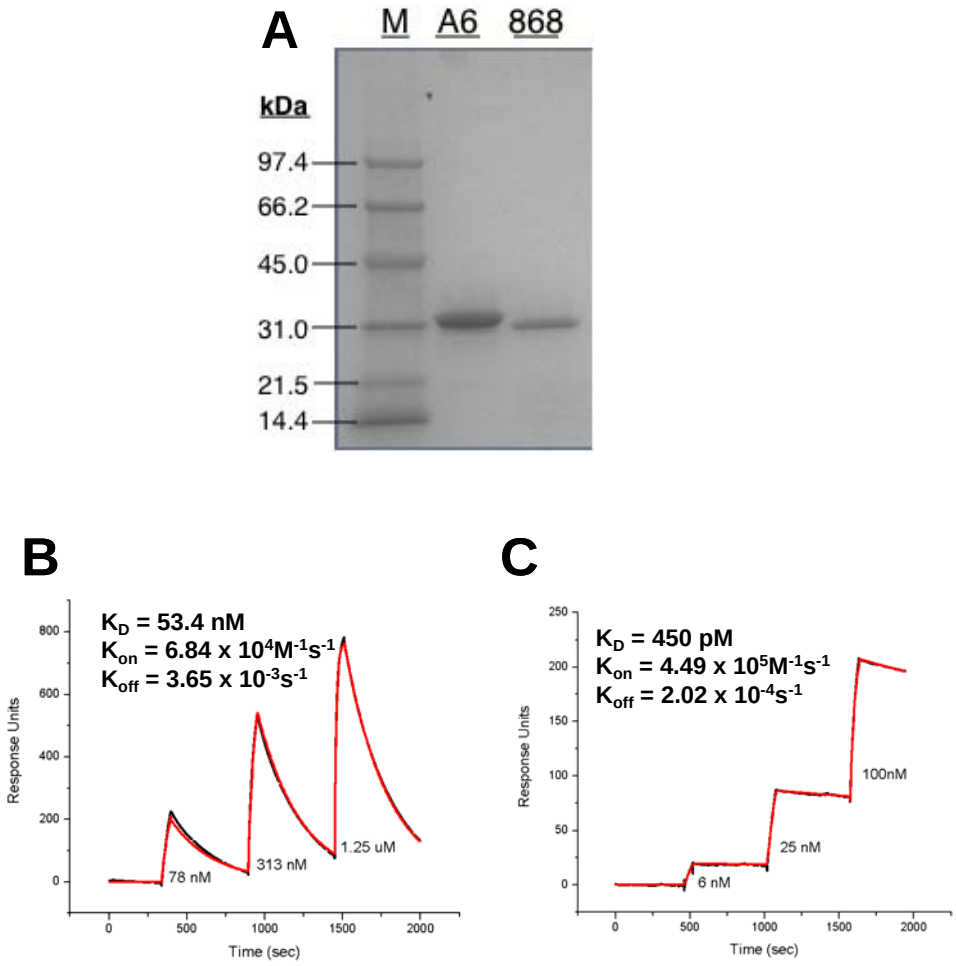

Figure 2.8. Expression, Purification, and Surface Plasmon Resonance Binding Studies of Soluble scTv Proteins A6-X15 and 868-Z11. A6 clone X15 and 868 clone Z11 were expressed in the *E. coli* pET28 expression system. Proteins were refolded from inclusion bodies, and purified by Ni-column and size exclusion chromatography. **(A)** SDS-PAGE of purified A6-X15 and 868-Z11 scTv proteins, and indicated molecular weight markers (M). **(B)** Surface plasmon resonance (SPR) traces of Tax/A2 binding immobilized A6-X15. Concentrations of injected pepMHC analyte are indicated below each injection. Red line represents a fit to a 1:1 binding interaction using a kinetic titration scheme as described in the Methods. Fitted parameters (K_D, k_{on}, k_{off}) are shown in the insert. **(C)** SPR traces of SL9/A2 binding immobilized 868-Z11. Experiments in B and C were performed by Kurt Piepenbrink and Francis Insaidoo in Professor Brian Baker's Lab (U. Notre Dame).

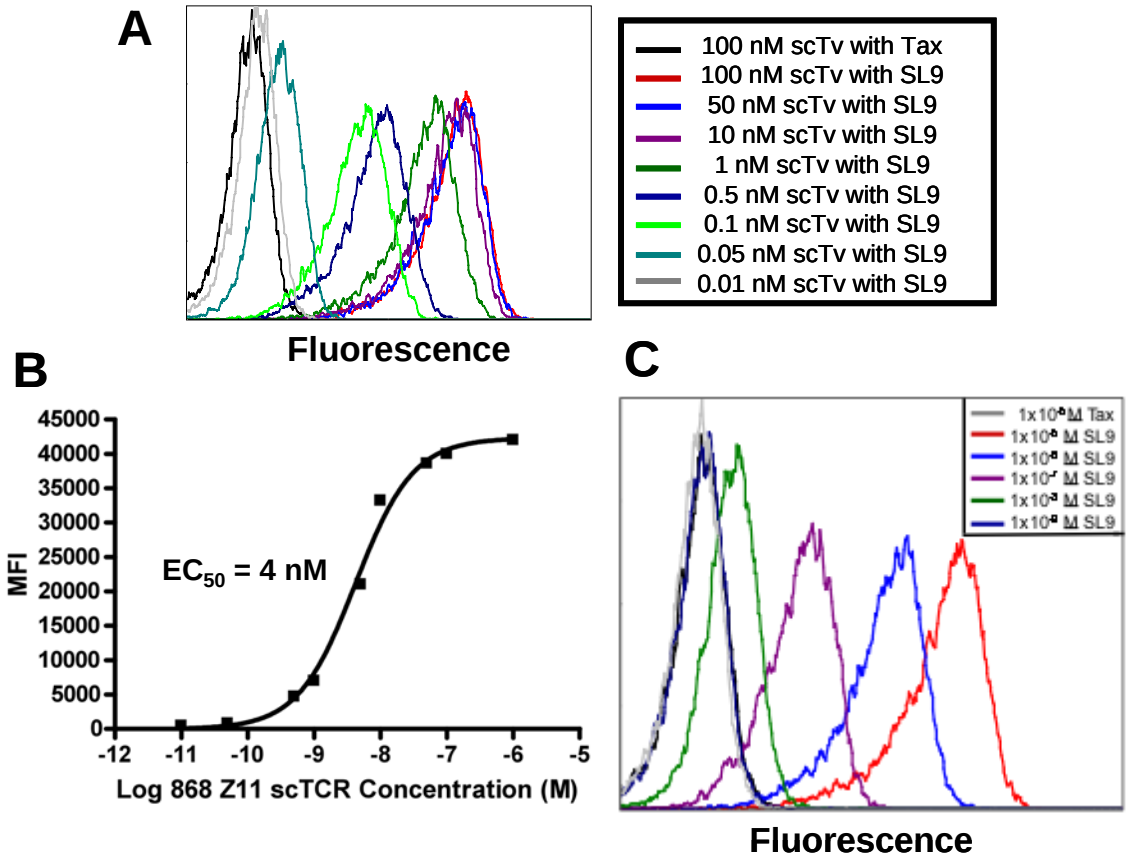

Figure 2.9. Recognition of Peptide-Loaded Antigen Presenting Cells with Soluble 868-Z11 scTv. (A) Titration of various concentrations of the biotinylated 868 scTv on antigen presenting cell line T2 (HLA.A2^{+}) pre-loaded with SL9 (10 μM) peptide, or null peptide Tax (10 μM). Cells were stained at room temperature for 30 min with scTv, washed, and stained with SA:PE for 30 min. Analysis was performed after two additional washes. **(B)** Mean fluorescence intensity (MFI) values of scTv titration shown in (A) were plotted versus scTv concentration, to determine the concentration that yielded 50% maximal binding, EC_{50}, for 868 Z11 scTv. **(C)** The SL9 peptide was titrated on T2 cells, and subsequently stained with a saturating concentration (100 nM) of biotinylated 868-Z11 scTv.

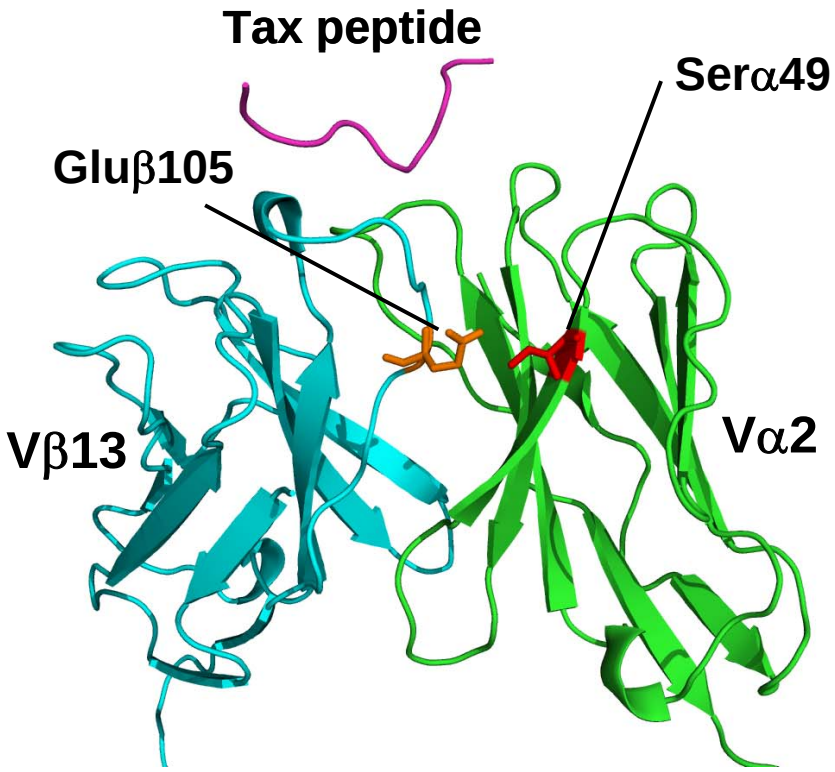

Figure 2.10. Position of the Serα49 Residue in the Structure of the A6 TCR. V regions of the A6:Tax/A2 complex (174) are shown, highlighting the position of the Serα49 residue which is 2.8Å from CDR3β residue Gluβ105. The Tax peptide (magenta) is shown for reference. PDB file 1AO7 was used in PyMol for graphics.

CHAPTER THREE

TRANSDUCTION OF SINGLE-CHAIN T CELL RECEPTORS LACKING CONSTANT REGIONS CIRCUMVENTS MISPAIRING WITH ENDOGENOUS TCR

Introduction

The potential of T cells in eliminating cancer cells has been demonstrated in numerous studies. In patients with clinically apparent cancer, however, T cell immunity has clearly failed to effectively reject the malignancy. Active immunization is being attempted to make the host's own T cells recognize and destroy its cancer, but this approach is complicated by the fact that T cells specific for the cancer cells, particularly those with high avidity or affinity, may be suppressed or have been deleted by central or peripheral tolerance. To improve the T cell response to cancer, recent approaches have combined adoptive T cell transfer with TCR gene therapy, in which a TCR of desired specificity and affinity is transduced into a patient's *ex vivo* activated T cells (196). This approach shows evidence of therapeutic promise (131,132) but also carries potential dangers (135).

T cells recognize foreign antigens, in the form of peptides bound to products of the major histocompatibility complex (pepMHC)[1], through a heterodimeric T cell receptor (TCR) consisting of α and β chains (156). A recognized complication of TCR gene therapy is the ability of introduced TCR α and β chains to mismatch with endogenous T cell receptors (197). In two recent studies, TCR mispairing between introduced and endogenous TCR α and β chains led to undesirable reactivities. In the first study using a mouse model system, overt autoimmunity (graft versus host disease) was observed, to varying degrees, in five different TCR systems (135). The other study with human T cells showed that mismatched heterodimers caused off-target toxicity, with an estimate of one of every ten mixed TCR heterodimers causing neoreactivity (198). These findings reinforce the idea that TCR mispairing could have detrimental, potentially dangerous, consequences.

A number of approaches have been developed to minimize the extent of mispairing (reviewed in (137, 197)). These have included the use of hybrid human TCR chains containing mouse constant domains, which associate preferentially with each other rather than with the endogenous TCR chains containing human constant domains (199). Another strategy involved fusion of the TCR chains to CD3ζ transmembrane and signaling subunits, also leading to preferential pairing of the exogenous TCR chains (200). Finally, several labs have shown that the substitution of non-native cysteine residues within the constant domains of the introduced TCR (160) also promotes pairing of the exogenous chains, presumably through the formation of a disulfide bond at the Cα:Cβ interface (201-203), significantly reducing the extent of mispairing and graft versus host disease in mouse models of TCR gene therapy (135). However, the full extent to which these strategies can prevent TCR chain mispairing is unclear.

An additional challenge of TCR gene therapy approach is that the levels of exogenous αβ TCRs expressed on the surface of T cells is reduced by the concurrent expression of endogenous αβ TCRs, since the total surface levels of TCR are controlled by the availability of the CD3 subunits, especially the ζ subunits (204, 205). As CD3 subunits are limiting, even the optimized pairing strategies described above will necessarily still result in lower exogenous TCR surface levels compared to a single, homogenous αβ TCR. Because TCR surface levels directly impact antigen sensitivity (206), it would be useful to develop strategies that enhance the levels of the exogenous TCRs.

Various single-chain TCR chimeras have been used in attempts to limit the problems associated with pairing of endogenous TCRs. These included three-domain TCRs that contain other signaling domains such as the CD3ζ intracellular domain (VαVβCβCD3ζ) (207-209) and three domain constructs along with a separate Cα domain (210). While these constructs mediate antigen-specific T cell activity, the extent of TCR mispairing and the quantitative comparisons of peptide activity with conventional two-chain αβ T cells has often not been examined. Single-chain T cell receptors consisting of only the TCR variable domains (VαVβ) in principle would

completely eliminate mispairing, but have been problematic due to instability of Vα and Vβ domains in the absence of constant domains (86).

Here I show that three domain TCR constructs (VαVβCβ) yield mispaired receptors in the presence of an endogenous α chain because of the contained Cβ domain, but stabilized two domain TCRs (VαVβ) with chimeric signaling domains avoid mispairing altogether and mediate T activity. The high-affinity scTv linked to intracellular signaling domains, Lck and CD28, was capable of activating T cells in the absence of either the CD3 subunits or the co-receptors CD4 or CD8. The scTv system also worked with a human construct specific for an HLA.A2–restricted peptide derived from the HIV-Gag protein. Our novel approach preserves the endogenous TCRs to potentially be triggered via intentional immunization or homeostatic proliferative signals, while permitting the introduced scTv TCRs to respond to the targeted antigen independent of CD3 subunits, without the transduced T cells acquiring any new undefined and potentially dangerous specificities.

Materials and Methods

Peptides, Antibodies, and Cell Lines

Peptides SIY (SIYRYYGL), OVA (SIINFEKL), SL9 (HIV-Gag, SLYNTVATL), Tax (LLFGYPVYV) and WT-1 (RMFPNAPYL) peptides were synthesized by the Macromolecular Core Facility at Penn State College of Medicine (Hershey, PA). Peptides were purified by reverse-phase chromatography using a C18 column with mass confirmed by MALDI.

The following antibodies were used: PE-conjugated anti-mouse CD3ε (BD Pharmingen, Clone 145-2C11), anti-mouseVβ8.2 (Clone F23.2), 1B2 clonotypic antibody, anti-mouse Vβ10 (Clone B21.5, eBiosciences), DimerX D^b (BD Biosciences), PE-conjugated anti-mouse CD8α (Clone 53-6.7, BD Pharmingen), goat anti-Mouse IgG AlexaFluor 647 (Molecular Probes) and anti-human Vβ5.2 (Clone 1C1, Pierce/Thermo Scientific). The 2C clonotypic antibody 1B2 was purified from hybridoma supernatant by ammonium sulfate precipitation followed by Protein G

chromatography (211). Biotinylation of purified antibody was performed using the EZ-Link Suflo-NHS-LC-Biotinylation Kit (Pierce).

The PLAT-E (Clonetech) retroviral packaging cell line was maintained in DMEM with 10% FCS, L-glutamine, penicillin and streptomycin. T2-K^b, a TAP-deficient cell line that can present exogenous peptides, and $58^{-/-}$ T cell hybridoma were maintained in RPMI 1640 complete medium supplemented with 10% FCS, L-glutamine, penicillin and streptomycin. The $58^{-/-}$ T cell line was transduced with retroviral supernatants of the full-length or scTv constructs as described(102).

Three-Domain and Two-Domain (scTv) Constructs

Various TCR constructs (Figure 3.1) were cloned into a murine stem cell virus (MSCV) retroviral vector (102). The two- and three-domain 2C and/or m33 single-chain TCR constructs contained six stabilizing mutations identified by yeast display (Vβ: Glu17Val, His47Tyr, Leu81Ser, Vα: Leu43Pro, Trp82Arg, Ile118Asn) (164, 165). 2C TCR contains Vα3.1 (TRAV9-4) and Vβ8.2 (TRBV13-2) gene segments. The three-domain TCR gene was codon optimized (Genscript) in the Vα3-linker-Vβ8.2Cβ orientation with a non-native cysteine at position 57 of the β chain constant region (Cβ: Ser57Cys), and introduced into the MSCV vector through AgeI/MluI restriction sites. For generation of the m33 Vα-linker-VβCβ-IRES-2CVαCα construct, the m33 three-domain construct was introduced into the 2C MSCV vector in the AgeI and XhoI restriction sites, replacing the 2C VβCβ chain (102).

For generation of scTv chimeric antigen receptor constructs, the murine CD8α hinge, CD28, CD3ζ, and LCK gene fusion was codon optimized (Genscript), based on sequences from the NIH database (GenBank accession #AAS07035.1) (208). The CD8α hinge was derived from scFv chimeric antigen receptor constructs previously described in murine systems (212), and the m33 scTCR (Vα-linker-Vβ) amplified by PCR from the three domain construct with a flanking 5' AgeI restriction site. Subsequently, the scTv was fused to the CD8α hinge, CD28, CD3ζ gene by overlap extension PCR with a primer that added a 3' MluI restriction site. The scTv gene fusion was introduced into the AgeI and MluI restriction sites of the MSCV vector. A

XhoI site exists at the 3' end of the m33 scTCR, prior to the start of the CD8α hinge region, allowing for introduction of other scTv genes with appropriate leader sequences into the scTv CD28,CD3ζ,LCK fusion protein. The 2C scTv with wild type CDR3α sequence (GFASA) was created by overlap extension PCR, and introduced into AgeI and XhoI sites in the m33 scTv construct.

The human TCR 868 against the HIV Gag peptide called SL9 (149) was synthesized as a single-chain Tv. The 868 scTv gene was amplified by PCR from the pCT302 yeast display vector, with a 5' primer that contained the leader sequence of the 2C scTv and an AgeI site, and a 3' end primer which included a XhoI site. The 868 scTv was then introduced into the m33 scTv vector containing CD8α hinge, CD28, CD3ζ, LCK fusion in MSCV by the AgeI and XhoI sites, with the 868 scTCR replacing the m33 scTCR in the fusion construct. The 868 scTv gene contained high-affinity CDR mutations derived from phage-displayed, full-length TCR (149).

T Cell Hybridoma Transductions

scTv and full-length TCR genes in MSCV were introduced into the PLAT-E packaging line to produce cell supernatant containing retrovirus for introduction into $58^{-/-}$ T cells. The retroviral packaging line was transfected with 40 μg of MSCV DNA with the lipofectamine 2000 reagent (Sigma) in Optimem serum-free media (Gibco). After four to six hours, cells were washed with supplemented DMEM and placed in 6 mLs supplemented RPMI. Forty-eight hours later, supernatant was collected, filtered, and added to $58^{-/-}$ T cell hybridoma cells with lipofectamine at a final volume of 8 μl/mL. T cells were centrifuged in retroviral supernatant at 1200 x g for 45 min at room temperature, cultured at 37°C for 3 days, and stained for cell surface mouse Cβ, mouse Vβ8, or human Vβ5 (868 scTv) with antibodies and analyzed on an Accuri C6 flow cytometer. To enrich for the transduced, positive population, cells were sorted with either anti-mouse Vβ8 (F23.2) for 2C and m33 or anti-human Vβ5.2 for 868, using a FACS Aria (BD Biosciences).

The 3D high-affinity TCR contained mouse Vα3 (TRAV9-1 subfamily, distinct from TRAV9-4 of 2C TCR, also called Vα3.1), mouse Vβ10 (TRBV4), and

CDR3 mutations that conferred higher affinity for WT-1/D^b (unpublished data), after isolation by T cell display (147). The 3D receptor contained unmodified TCR constant domains (Cα48:Thr, Cβ57:Ser), and was introduced into the $58^{-/-}$ line as described for scTv and three-domain constructs. The 3D TCR^+ population was sorted with anti-Cβ antibodies prior to transduction of m33 scTv or m33 three-domain.

PepMHC Multimer Binding

Transduced cells were stained with SIY/K^b pepMHC tetramer on ice for 2 hours. SIY/K^b tetramer was produced as has been described (102). After washing, cells were resuspended in cold PBS/BSA and analyzed for bound fluorescent tetramers by flow cytometry. Non-transduced T cell hybridoma $58^{-/-}$ line was used as a control. Alternatively, WT-1/D^b pepMHC Ig-dimers (DimerX, BD Biosciences) were prepared by incubation overnight at 37°C with WT-1 peptide at 120-molar excess. Transduced cells were stained with WT-1/DimerX for 2 hours on ice in the dark, washed twice with 10-fold excess volume PBS/BSA (0.5%), and incubated on ice for 1 h in the dark with goat anti-mouse IgG APC Fab' Alexa Fluor 647 at 10 μg/mL (Molecular Probes).

T Cell Activation Assays

T cells (7.5×10^4) were incubated with SIY or OVA peptide loaded T2-K^b cells (7.5×10^4), plate bound antibodies (anti-CD3ε or anti-mouse Vβ8 (F23.2)), or plate bound pepMHC tetramer (SIY/K^b or OVA/K^b) as described (102). For the 868 scTv cell line, cells were incubated with plate-bound antigenic SL9 (HIV Gag)/HLA.A2 or null Tax/HLA.A2 streptavidin tetramers or SL9 or WT-1 peptide-loaded T2 cells (7.5×10^4). T cells were incubated with the various stimuli for 24 h at 37°C in a final volume of 200 μL, and supernatants were collected. For IL-2 detection, 96-well plates were coated with 2.5 μg/mL anti-murine IL-2 (BD Pharmingen) in 0.1 M Na_2HPO_4 (pH=9.0), and IL-2 in supernatants was detected with 6.7 μg/mL biotinylated anti-murine IL-2 (BD Pharmingen), followed by a 1/10,000 dilution of streptavidin-HRP (BD Pharmingen), and finally, TMB substrate

(Kirkegaard & Perry Laboratories). Absorbance at 450 nm was measured with an EL_x800 universal plate reader (Bio-Tek Instruments).

Results

Three-Domain Single-Chain TCR (VαVβCβ) Containing a Non-Native Cβ Cysteine Mispairs with Endogenous TCR Chains

Various studies have suggested that a single-chain TCR construct consisting of tandem VαVβCβ domains could minimize the potential for mispairing with endogenous TCRs. Inclusion of a non-native cysteine in C regions represents another strategy to minimize such mispairing. Since the Cα:Cβ interaction contributes significant binding energy to these pairing interactions, I decided to explore whether a single-chain TCR (VαVβCβ) that contains a non-native Cβ cysteine would mispair with an endogenous α chain (VαCα), presumably through the interaction of the Cβ with the Cα. To directly assess this, I took advantage of high-affinity probes for two different, properly associated VαVβ pairs. The first probe, for the 2C TCR, is a clonotypic antibody, denoted 1B2, that binds only to properly associated 2C VαVβ with an affinity of 1 nM (213). The second probe, for the mutated 2C TCR denoted m33 TCR, is the SIY/K^b ligand that binds only to properly associated m33 VαVβ with an affinity of 30 nM (101). The mutations that convert 2C to the high-affinity m33 reside in the Vα CDR3 region, and prevent binding of 1B2 (147). In addition, since the affinity of the SIY/K^b complex for 2C is low (30 μM), this tetramer bound by m33 only binds to the 2C TCR in the presence of CD8 (214).

Expression of the m33 three-domain (VαVβCβ) construct (Figure 3.1) in the T cell hybridoma $58^{-/-}$ resulted in cells that were cell surface positive for Cβ and weakly positive for staining with SIY/K^b (Figure 3.2A), but as expected were negative for staining with 1B2. The vector containing the m33 three-domain construct was then transduced into $58^{-/-}$ cells together with the α chain from 2C (VαCα). These cells were not only positive for anti-Cβ and SIY/K^b, but were

strongly positive for 1B2. The only way for the 2C α chain to reconstitute the 1B2-binding site is for the VβCβ region of the m33 three-domain construct to associate with the 2C α chain on the surface. Thus, the three-domain construct with the non-native Cβ cysteine did not prevent pairing with the endogenous α chain lacking a non-native Cα cysteine.

To verify that the three-domain construct was capable of mispairing with another TCR, when both the α and β chains were expressed, I used a mouse TCR called 3D that is specific for WT-1/D^b. This TCR included mutations that improve the binding to WT-1/D^b, as engineered by T cell display (unpublished results). The 3D TCR allowed detection of binding to a WT-1/D^b dimer in the absence of co-receptor CD8, and thus, like m33 the pepMHC can be used directly to probe for properly associated 3D αβ. The 3D TCR was transduced with unmodified constant domains (Cα48:Thr, Cβ57:Ser) in order to provide a direct test of pairing of the three-domain m33 construct with the endogenous TCR chains. The m33 three-domain construct was then introduced into the 3D TCR expressing $58^{-/-}$ T cells and cells were sorted with an antibody to Vβ8 (m33). Surface levels of (i) m33 three-domain construct alone, (ii) 3D TCR construct alone, or (iii) the combination of 3D TCR and m33 three-domain constructs were examined with anti-mouse Vβ10 (3D TCR), WT-1/D^b Ig dimer (3D TCR), and anti-mouse Vβ8 (m33). In cells co-expressing the m33 and 3D constructs, binding to the 3D ligand WT1/D^b was significantly reduced, and binding to the m33 Vβ was significantly increased (Figure 3.3). These results would appear to reflect mispairing of the m33 three-domain construct with the 3D α chain, resulting in a decrease in 3D heterodimers and the associated reduction of binding WT1/D^b, and an increase in m33 Vβ expression from mispairing with the 3D α chain. The coexpressed 3D TCR and m33 three-domain construct showed similar levels of Vβ10 as the m33 three-domain construct alone potentially because of ββ homodimers, as observed by others expressing murine constant domains (203).

Expression of a scTv (VαVβ) Avoids Mispairing

As the three-domain construct mispaired with endogenous TCR chains, I reasoned it might be possible to use a single-chain TCR that lacked a Cβ region in order to completely avoid mispairing. Similar approaches have used scFv fragments as chimeric antigen receptors (CAR), consisting of various fused signaling domains (155). Although analogous single-chain TCR molecules are typically unstable without the Cβ domain, I have shown that it is possible to engineer scTv (VαVβ) by incorporating stabilizing mutations in the V regions (86, 154, 164).

To test this strategy, I cloned the stabilized scTv of m33 as a fusion with CD28, CD3ζ, and Lck domains. The m33 scTv fusion was introduced into $58^{-/-}$ alone or with the 3D TCR and expressed at high levels on the surface of the $58^{-/-}$ line, as detected with both the anti-Vβ8 antibody and the SIY/K^b (Figure 3.4). The binding of SIY/K^b indicated that the Vα and Vβ domains were associated appropriately at the cell surface. Furthermore, the expression of the m33 scTv was not influenced by expression of the 3D TCR, nor was the 3D TCR surface levels affected by the m33 scTv. These results suggest that significant mispairing was not occurring.

Surface Levels of the Stabilized scTv are Higher Than Conventional TCR

To determine whether the surface levels of the scTv fusions of the TCR were comparable to the conventional αβ TCR/CD3 complex, I examined transduced cells expressing the m33 scTv fusion or the full length m33 αβ chains in the absence of endogenous TCRs or the CD8 co-receptor. The scTv fusion was expressed at approximately five-times the surface level (based on mean fluorescence intensities) of the full-length αβ TCR, as detected with anti-Vβ8 antibody (Figure 3.5A). The wild type, low affinity 2C TCR expressed as either a scTv fusion or in the full-length form was detected at nearly identical levels to m33 (Figure 3.5A).

Since typical binding studies with pepMHC tetramers take advantage of the avidity achieved through the presence of many TCRs per cell, I next examined the binding of SIY/K^b tetramers to low (2C) and high-affinity (m33) forms of the TCR expressed as either a scTv fusion or full αβ TCR. As previously described (102, 214,

215), the αβ full-length, high-affinity TCR m33, but not the 2C, was capable of binding tetramers in the absence of CD8. The higher surface levels of the scTv forms yielded enhanced staining with the SIY/K^b tetramer, including significant binding by the 2C scTv despite its relatively low affinity (K_D = 30 μM). Full titrations with SIY/K^b tetramer revealed that the 2C low affinity scTv bound even higher levels than the high-affinity m33 expressed as a conventional αβ TCR, despite the 1000-fold higher affinity of m33 (Figure 3.5C). This finding highlights the importance of surface densities have on the binding of multimeric ligands.

*Efficient T Cell Activation is Mediated by the Stabilized scTv Fusion*s

To examine the activation of T cells mediated by the scTv fusions, I examined IL-2 release by the transduced $58^{-/-}$ lines using various stimulating ligands. The full-length TCR was assembled with CD3 subunits, whereas surface expression of the scTv did not require nor sustain CD3 as evidenced by the absence of staining with the anti-CD3ε antibody (Figure 3.6A). Despite the absence of CD3ε from the surface of cells transduced with scTv fusions, these cells were efficiently stimulated by anti-Vβ antibody immobilized on plates (Figure 3.6B). Cells that expressed the conventional αβ TCR were also stimulated by immobilized anti-CD3ε, whereas the scTv cells were not simulated by anti-CD3 antibodies (Figure 3.6B).

Antigen-presenting cells (T2-K^b) were used in the presence of various concentrations of the SIY peptide to determine if cells transduced with different forms of the TCRs mediated peptide dependent, CD8-independent activity. As described previously (101, 102, 216), $CD8^-$ cells transduced to express the m33 full-length TCR, but not the 2C TCR, responded to SIY peptide in the presence of T2-K^b (Figure 3.6C). The m33 scTv fusion stimulated activity at approximately the same concentration of peptide as the full-length m33 αβ TCR. In contrast, the 2C scTv was not stimulated by SIY-loaded T2-K^b cells, despite the high surface levels of the scTv form and the ability of the scTv to bind to SIY/K^b multimers (Figure 3.5C). Thus, in the absence of CD8, avidity did not overcome the requirement for an inherent binding threshold (102). However, immobilized SIY/K^b tetramers were capable of stimulating the 2C scTv fusions to a greater extent than the T2-K^b

presented antigen (Figure 3.6D), consistent with our previous observations that low affinity TCRs can be stimulated when the antigen densities are raised above a level achievable on APCs.

T Cell Activity Mediated Through a Stabilized Human scTv Fusion

To examine the applicability of the scTv system to another TCR, a human TCR denoted 868, specific for HIV-Gag/HLA.A2 (149), was introduced as an scTv construct. A stabilized scTv mutant of the parental αβ 868 TCR was engineered by yeast display (unpublished results). The 868 scTv exhibited high-affinity for HIV-Gag (SL9)/HLA.A2 (K_D < 2 nM)(data not shown). The human 868 scTv was fused to the same mouse intracellular signaling domains (Figure 3.7A) as with the 2C and m33 systems, the construct was transduced into the $58^{-/-}$ T cell hybridoma, and the 868 scTv was detected on the surface as monitored by anti-human Vβ5.2 antibody (Figure 3.7B). The 868 scTv cell line was activated by SL9 peptide presented by T2 cells and by plate-bound SL9/HLA.A2 tetramer, but not the null peptide ligands WT1 or Tax (Figure 3.7C,D), consistent with our results with other TCR chains. This experiment shows that scTv chimeric receptors with antigen specificity can be generated from other TCRs, and that the strategy can be extended to human epitopes.

Discussion

Recent studies have introduced virus- or cancer-specific TCRs into T cells as a strategy to provide a patient with an enhanced T cell response, and ongoing studies are attempting to optimize this approach (131, 137, 196, 197, 217). In a related strategy to provide tumor-reactive T cells, transduced T cells expressing chimeric antigen receptors (CARs) that contain a scFv fused to intracellular T cell signaling domains (e.g. CD28, CD3ζ, and Lck) have entered clinical testing (155). Combining these approaches, several labs have attempted to use single-chain, three-domain TCRs (VαVβCβ) that can mediate proximal signaling through fused intracellular signaling domains (207, 208, 218). In principle, the advantages of these TCR fusions are that a single gene product could recognize target cell pepMHC antigens and stimulate the

effector functions associated with T cells, independent of endogenous CD3 molecules (137). However, the three-domain strategy has been limited in some instances because the TCR surface levels are insufficient for recognition of low levels of antigen (209). Furthermore, while the three-domain TCR construct provides the stability necessary for surface expression, I show here that it does not avoid a major problem associated with use of exogenous TCRs in gene therapy, mispairing with endogenous TCR chains. This mispairing has recently been shown to result in neoreactivities (198), and T cell-induced graft-versus-host disease in mouse models (135).

Improvements in surface levels of exogenous TCRs have been achieved by various methods, including codon optimization to enhance transcription levels (219), regulation of αβ chain stochiometry to improve proper pairing (220, 221), inclusion of non-native cysteines (201, 202), modification of other residues in the C regions (222-224), substitution of mouse C regions in human TCRs (199, 225, 226), or direct fusion of CD3ζ domains (200) to drive desired pairing of the introduced chains. However, the diversity of αβ TCRs makes it unlikely that mispairing can be completely avoided using these approaches. Therefore, attempts to avoid the problem altogether have involved use of different recipient cell types such as γδ T cells (227-229), NK cells (230), or oligoclonal populations of αβ T cells (135, 231). However, these approaches also have limitations, including difficulty isolating and transducing a sufficient numbers of cells for adoptive transfer. A method to silence endogenous TCR has been developed using siRNA, but the duration and efficiency of endogenous TCR suppression is unclear (232). It is worth noting that because CD3ζ subunits are limiting in a T cell (205), several of these approaches still lead to competition between the exogenous and endogenous TCRs for CD3, resulting in lower levels of both receptors.

Our results show that the inclusion of a Cβ region with a non-native cysteine allowed association with endogenous α chains, and that deletion of the Cβ effectively eliminated pairing, within the limits of detection by flow cytometry (i.e. approximately 1,000 molecules/cell). The ability of α and β chains to pair is dependent on the interactions of the Vα:Vβ regions and the Cα:Cβ regions, but the

relative contributions of each are not known. Assembly of the entire αβ/CD3 complex requires charged residues in the transmembrane domains of the various subunits (104). While the association of the Cα and Cβ domains presumably is the same among different TCRs, the Vα:Vβ interaction varies, and is controlled by multiple residues at the interface including regions within the CDR3α and CDR3β (86, 233). Hence, TCR chain pairing studies often reveal "strong" or "weak" TCRs; and the efficiency of a particular αβ association helps determine the surface levels of a TCR pair in cells expressing multiple α and β chains (217, 234, 235). Our data demonstrates that a TCR with Vα and Vβ regions alone is insufficient to drive detectable association with an endogenous α and β TCR chains, suggesting that the dimerization constants of the V regions is low, and a large fraction of the association energy is driven by interactions between Cα and Cβ regions (210). As with the original principle of linking V_L and V_H to form scFv fragments (236), the scTv provides a strategy for forcing the association between the linked domains. In practice, this is not trivial, and single-chain VαVβ fragments (scTv) are considerably less stable than scFv fragments (86). Our lab has previously overcome this obstacle by engineering scTv with key stabilizing mutations in the V regions (86, 154, 164), and here I show that these scTv can be generated as fusions with intracellular signaling domains that can mediate T cell activation following antigen recognition. While chimeric antigen receptors directed by scFv fragments, sometimes referred to as T-bodies, have been useful in targeting non-MHC restricted cell surface antigens, the recognition of MHC-associated antigens by scTv fragments makes it possible to target the vast array of intracellular antigens invisible to scFv on viable cancer or virally infected cells. In addition, scTv can recognize antigens released by cancer cells and cross-presented by tumor stroma, which can be critical for eradicating antigen-loss variants in a tumor mass and thereby prevent relapse after therapy (138, 139). In principle, scFv fragments specific for MHC-restricted antigens could also be used for these purposes (237-240).

In order for a TCR, and presumably a scTv, to mediate activity in the absence of CD8 or CD4 binding to the presenting MHC molecules, its affinity needs to be

above a threshold of about 1 μM (102). Because of this threshold limitation, the wild type TCR 2C (K_D = 30 μM) did not mediate activity against cell-presented antigen either as a scTv, or as a full-length TCR in the absence of CD8 (Figure 3.6C). Optimization of the fused signaling domains may be able to enhance the sensitivity of scTv, or possibly even overcome issues associated with tolerance when T cells must function in a suppressive tumor environment (e.g. (241, 242)). Nevertheless, it still remains questionable whether, in the absence of CD8, scTv that have not been modified to increase affinity will be capable of targeting the pepMHC levels found in many cancer or virus-infected cells. Fortunately, TCRs with affinities similar to those of antibodies can be engineered (159), and using such an engineered TCR targeting the HIV Gag antigen (SL9)/HLA.A2 (149), I show that a scTv form of this receptor can mediate T cell activation. Such high-affinity scTv could be used in a TCR gene therapy format, without the risk of TCR mispairing that would be associated with receptors containing human or mouse Cα and Cβ regions, or their mutated variants. Furthermore, the higher affinity scTv would be capable of mediating the activity of $CD4^+$ T_H cells (147),(309).

Another potential advantage of the scTv-fusion approach is that the endogenous TCR/CD3 complexes remain at the same level on the transduced T cell as on the parental cell. In a study of T cells that expressed two transgenic TCRs, it was possible to overcome tolerance mediated by one of the TCRs through signaling mediated by the other TCR (241). It has also been shown that activation of T cells through the endogenous TCR can promote the persistence of transduced T cells, thereby enhancing their effectiveness against the target antigen of the transduced TCR (243). Thus, the ability of the scTv fusions to avoid mispairing could preserve efficient signaling through the endogenous TCR, and yet allow the introduced scTv-fusion to mediate re-directed activity against tumor or viral epitopes.

Figures

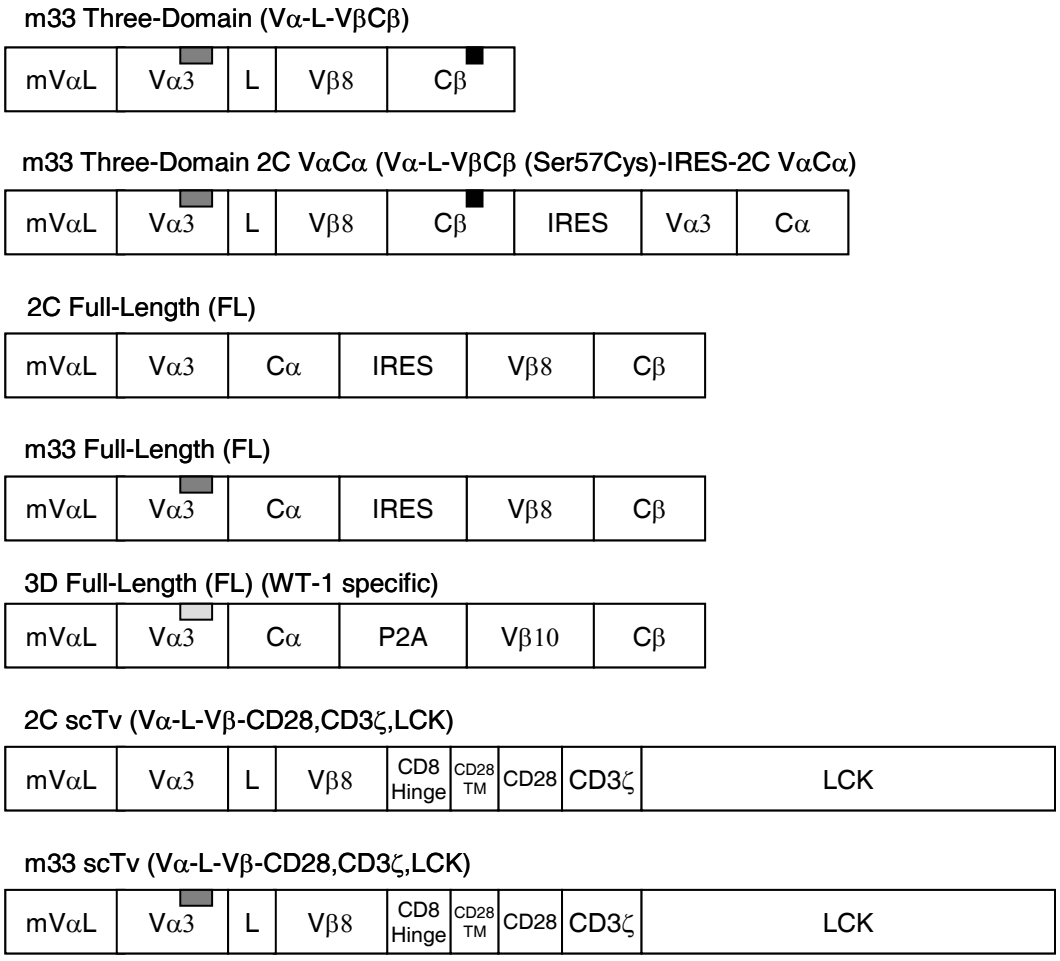

Figure 3.1. Full Length, Three-Domain, and Single-Chain VαVβ (Tv) TCR Constructs. TCRs derived from the 2C or 3D TCR were introduced into the murine stem cell virus (MSCV) retroviral vector. The m33 high affinity variant of the 2C TCR differs only in the CDR3α sequence: 2C CDR3α-GFASA, m33 CDR3α-LHRPA (designated by gray insert rectangle). The 3D full-length receptor also contained CDR3α mutations that confer higher affinity to WT-1/D^b. The m33 three domain constructs contained a non-native cysteine mutation (Cβ: Cys57), indicated by the black rectangle. IRES refers to internal ribosome entry site, CD28TM refers to the CD28 transmembrane domain, and mVαL refers to the murine Vα leader sequence.

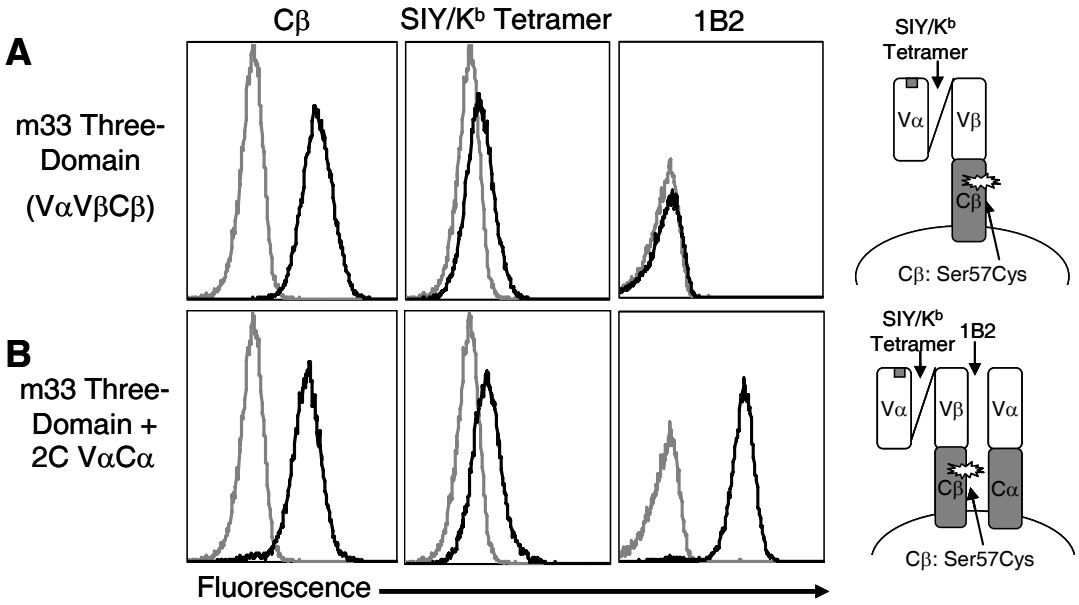

Figure 3.2. Three-Domain m33 TCR Mispairs with Endogenous TCR α Chains. T cell hybridoma $58^{-/-}$ mock (no vector DNA) transduced cells (gray line) and transduced cells (black line) were analyzed by flow cytometry for surface expression after transduction with **(A)** the m33 three-domain construct or **(B)** the m33 three-domain construct and the 2C full α chain construct (2C VαCα) transduced cells were examined with anti-Cβ antibody, SIY/K^b Tetramer (100 nM) or 1B2 clonotypic antibody. The m33 three-domain cassettes in both constructs contained a Cβ non-native cysteine while the 2C VαCα chain contained unmodified constant domains.

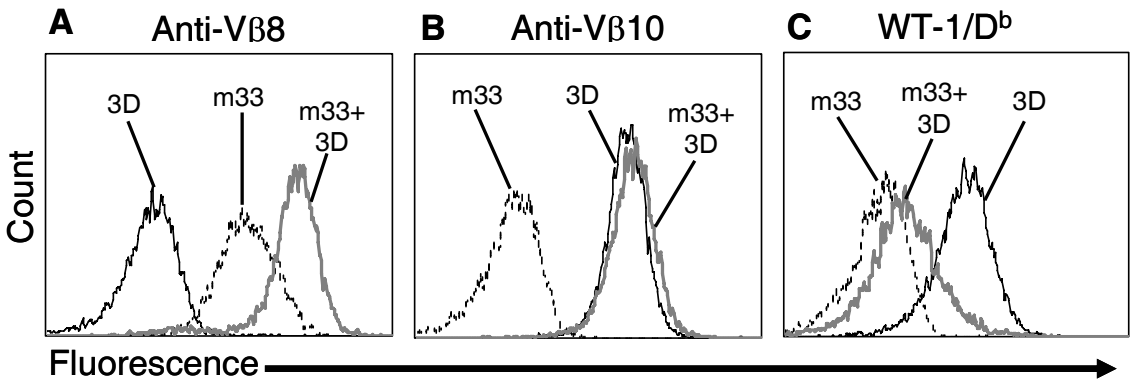

Figure 3.3 Three-Domain m33 TCR Mispairs with Endogenous TCR 3D. The WT-1 specific TCR 3D, which contains unmodified murine constant domains, was used to mimic an endogenous TCR (3D TCR: mVα3, mVβ10). 3D TCR-positive T cell hybridoma $58^{-/-}$ were transduced with the m33 three-domain TCR ($V\beta8^{+}$) construct. Vβ8-sorted cells were analyzed with anti-Vβ8 **(A)**, anti-Vβ10 **(B)** or WT-$1/D^{b}$ DimerX at 125 nM **(C)**. Transduced 3D cells that co-express the m33 three-domain construct (gray line) were compared to cells that express only the 3D full-length TCR (black line), or only the m33 three-domain construct (black dashed line).

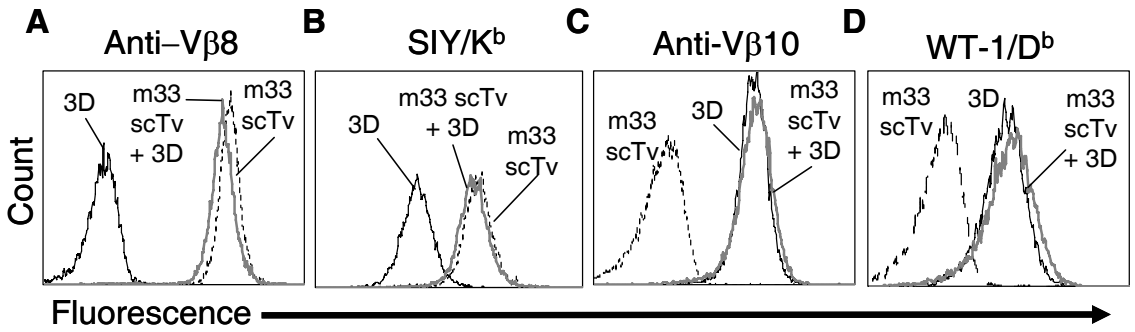

FIGURE 3.4. A Single-Chain VαVβ TCR (scTv) Construct Avoids Detectable Mispairing with Endogenous TCR 3D. 3D expressing T cells were transduced as in Figure 3, but in this instance with the m33 scTv. Following sorting with anti-Vβ8 antibody, cells were analyzed with anti-Vβ8 (A), SIY/K^b tetramer at 100 nM (B) anti-Vβ10 (C), or WT-1/D^b DimerX at 125 nM (D). Transduced 3D cells that co-express the m33 scTv construct (gray line) were compared to cells that express only the 3D full-length TCR (black line), or only the m33 scTv construct (black dashed line).

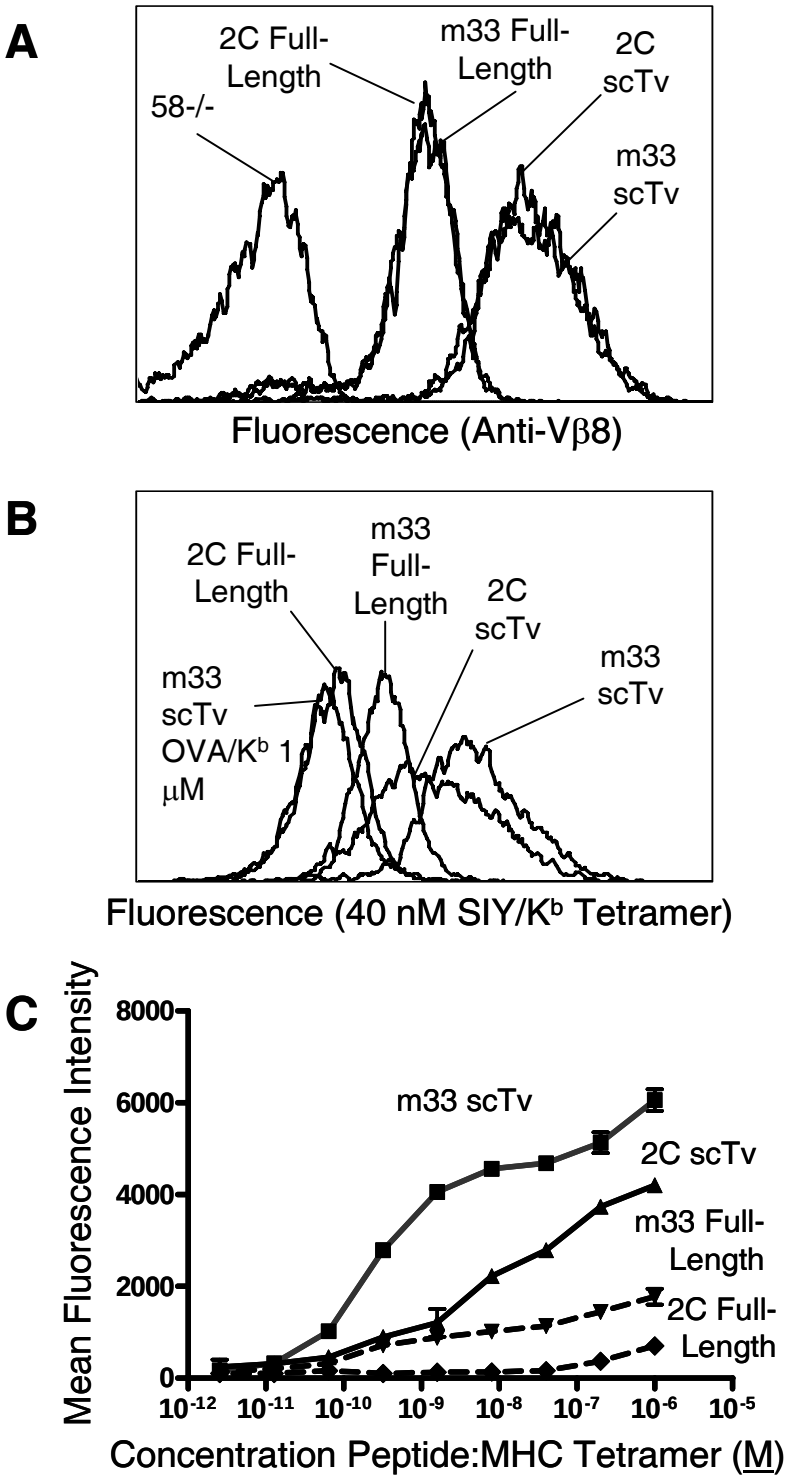

Figure 3.5. Surface Levels and SIY/K^b Binding by scTv and Full-Length TCRs. scTv and full-length 2C (K_D=30 μM for SIY/K^b) and m33 (K_D=30 nM for SIY/K^b) constructs were introduced into 58$^{-/-}$ cells. Cell surface expression was monitored with anti-Vβ8 **(A)** or SIY/K^b SA:PE labeled tetramer at 40 nM **(B)** by flow cytometry. SIY/K^b tetramer binding to scTv and full-length constructs was examined at various concentrations of tetramer **(C)**.

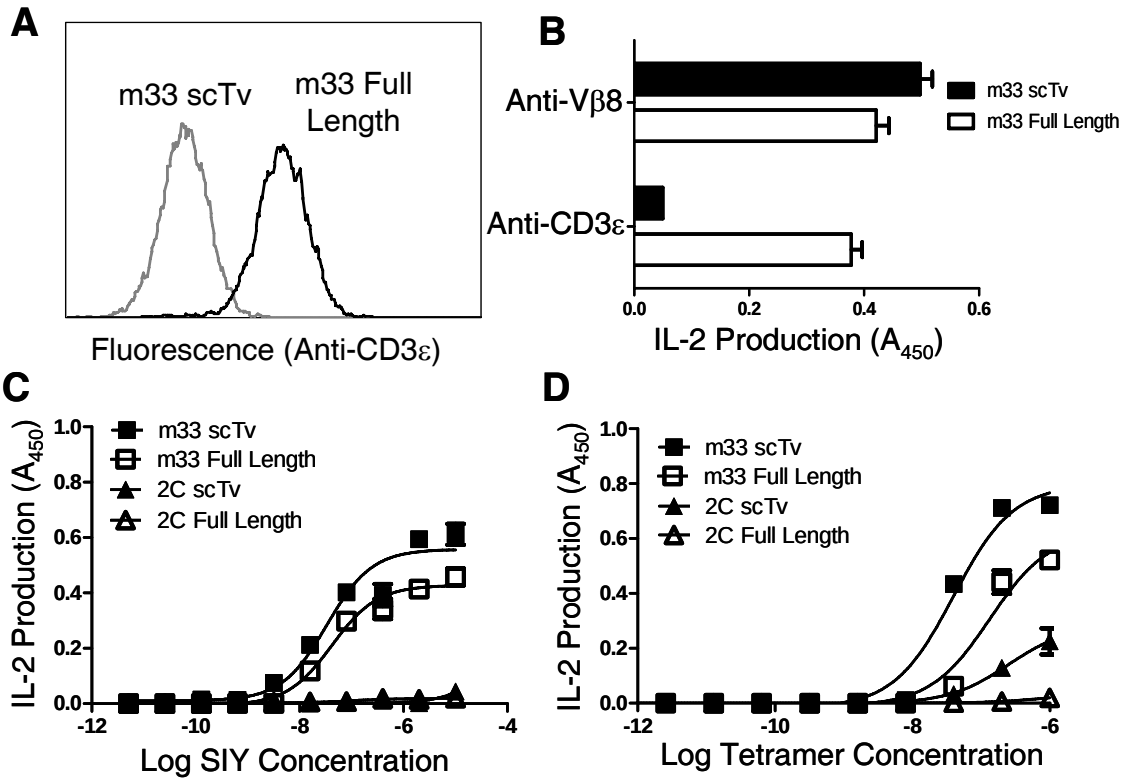

FIGURE 3.6. Comparison of m33 (High Affinity) and 2C (Low Affinity) Full-Length and scTv Constructs for Antigen Specific T Cell Activation. (A) CD3ε surface expression of the m33 scTv (gray line) and full-length TCR (black line). **(B)** Activation of IL-2 release from m33 scTv and full-length expressing cells with plate bound anti-Vβ8 or anti-CD3ε antibodies. **(C)** Antigen specific activation of 2C and m33 scTv and full-length transduced cells using peptide-loaded T2-K^b cell line at various concentrations of exogenous SIY peptide. **(D)** Antigen specific activation of 2C and m33 scTv and full-length transduced cells using plate bound SIY/K^b tetramer. Data for B, C, and D is representative of three independent experiments for each panel. OVA peptide (10 μM) loaded T2-K^b cells and OVA/K^b plate-bound pepMHC tetramer gave absorbance values from the IL-2 ELISA at background levels (not shown).

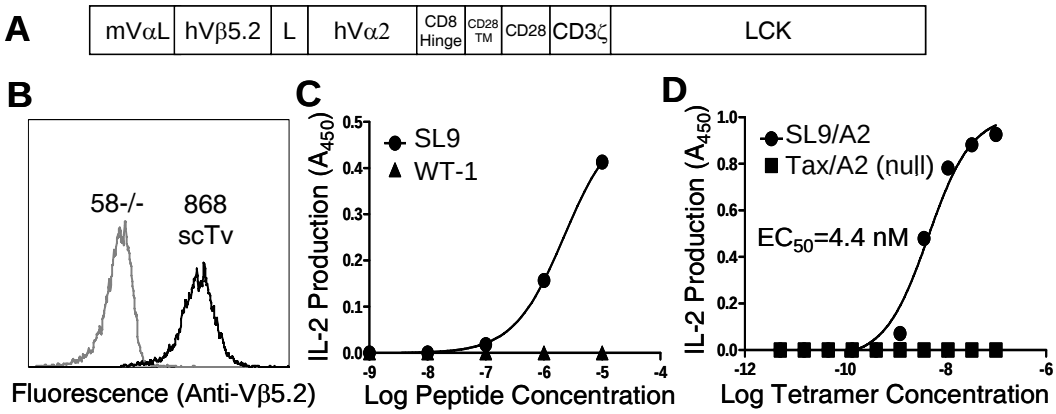

Figure 3.7. The Human High-Affinity HIV-Specific 868 scTv Mediates Antigen-Specific Activity in $58^{-/-}$ T Cells. (A) The leader from the 2C scTv was introduced upstream of the 868 scTv, and the human TCR was expressed as a fusion to murine intracellular signaling subunits. **(B)** Surface expression of 868 scTv in transduced cells was monitored by anti-human Vβ5.2. (gray line - $58^{-/-}$ cells, black line – 868 scTv). To assess antigen specific activation, 868 scTv expressing cells were stimulated with **(C)** SL9 or WT-1 (null) peptide loaded T2 cells or **(D)** plate-bound SL9/A2 or Tax/A2 tetramers at various concentrations. Data in C and D are representative of two independent experiments for each panel.

CHAPTER FOUR

COMPARISON OF SINGLE-CHAIN T CELL RECEPTOR (SCTV) AND ANTIBODY (SCFV) FRAGMENTS IN MEDIATING T CELL ACTIVITY

Introduction

One recent approach to tumor immunotherapy relies on targeting tumor associated antigens (TAAs) with antibodies. Monoclonal antibodies against TAAs are gaining widespread use in clinical cancer immunotherapy as more antigens and antibodies with defined specificity are identified (Reviewed in (141),(244), and (245)). For instance, clinical use of the monoclonal antibodies Herceptin and Rituximab has improved remission rates for breast cancer (246),(247),(248) and various hematological malignancies (249),(250) respectively. Some patients, however, demonstrate cancer progression after treatment with monoclonal antibodies due to low levels of antigen expression on cancer cells, or possibly due to inefficient antibody Fc-mediated destruction of the cancer cell. To improve efficacy, numerous groups are developing chimeric antigen receptors (CARs) consisting of an antigen-binding domain (single-chain fragment variable, scFv) fused to intracellular signaling domains to redirect T cells to a wide variety of tumor-associated antigens.

For example, patients with osteosarcoma often express low levels of HER2 surface protein such that tumors do not respond to treatment with Herceptin, a HER2-specific antibody. Introduction of scFv CAR expressing T cells directed at HER2+ osteosarcoma allowed for *in vivo* targeting of cells that expressed the low antigen levels (251). The identical scFv CAR from that study was subsequently used to target HER2+ glioblastoma, a tumor type that was previously resistant to antibody therapy (252). ScFv CARs introduced into T cells are currently being investigated in clinical trials because of the ability to rapidly convert an scFv already approved for soluble therapy into a CAR format, where the antigen-binding domain does not need to be altered, and both specificity and affinity are well-defined.

The scFv CAR approach provides a potential advantage for tumor targeting because target antigens are often expressed at high surface levels with relatively low

levels of expression on non-malignant tissue. Unlike T cell receptors (TCRs), the antigen for scFv is most often not MHC-restricted, allowing a larger population of patients to be eligible for treatment than by conventional T cell therapies. For example, some individuals lack common MHC alleles and consequently must be excluded from certain TCR-based therapies. A potential disadvantage of targeting non-MHC-restricted antigens, however, is that cross-presentation of antigen by tumor stroma can not occur, thereby preventing a major path by which tumors can be destroyed (i.e. MHC-restricted peptide antigens from tumors can be cross-presented on nearby support cells within solid tumors herein referred to as tumor stroma) (253,139,169). Thus, although peptide:MHC complexes may be present at lower surface levels relative to antibody-targeted antigens (a comprehensive list of MHC restricted tumor antigens reviewed in (134)), it may be necessary to target these MHC restricted antigens to destroy tumor stromal cells and thereby prevent tumor outgrowth.

In this work, I sought to compare the efficacy of an antibody scFv CAR with a T cell receptor based scTv (single-chain TCR variable) CAR to mediate antigen specific activity. A chimeric antigen receptor that is specific for a cell surface protein with a tumor associated mutation was generated by fusing the antibody scFv, called 237, to intracellular signaling domains. The 237 antibody was derived from an aging female mouse that had an immune response to fibrosarcoma (254, 255). The antigen recognized by 237 was subsequently shown to be due to a mutant chaperone protein, which resulted in the loss of activity associated with a glycotransferase. This in turn led to the expression of a neo-epitope consisting of the defective carbohydrate linked to a peptide on the tumor cell surface. The glycosylation defect recognized by the 237 antibody is specific for the tumor, as the post-translational defect is only present in the transformed cells.

The 237 epitope containing the glycosylation defect was originally isolated from the OTS8 transmembrane protein between amino acids 75 and 85 (RGTKPPLEELS), a sialomucin-like transmembrane glycoprotein that lacks a galactose group modification. Further study of the glycosylation defect revealed that this post-translational modification existed in many cell surface proteins that contain

similar amino acid sequences, and these epitopes are accessible to antibodies on the tumor cell surface (254). Notably, Tn antigens derived from a similar glycosylation defect, have been identified in breast cancer (256), gastrointestinal tract tumors (257), and metastatic melanomas (258). As a consequence, antibodies like 237 directed toward sugar moieties specific to tumor cells could provide clinical benefit against a broad range of human tumor types. The crystal structure of the 237 antibody in complex with the mutated glycopeptide revealed that 237 recognizes the carbohydrate moiety with relatively weak interactions directed to the peptide backbone (259). This finding is rare among glycopeptide-specific antibodies, which are most often directed against the peptide and as a result cross-react with non-malignant and embryonic tissue (260). The affinity of 237 for the glycopeptide has been measured (259), (K_D=140 nM) which is greater than wild-type TCR affinities for peptide:MHC (K_D ~ 1,000-500,000 nM). The 237 affinity for the native cell surface epitope has not been measured, and thus it may be even higher.

To assess the efficacy of the scTv approach, the 237 scFv CAR was compared with the high affinity m33 scTv (K_D=30 nM) (described in chapter 3) that targets the model tumor antigen SIY/K^b (102). In the first half of this chapter, I compare the 237 scFv CAR and the m33 scTv CAR in targeting antigen-positive tumor cells, and I assess the contribution of the CD8 coreceptor in scFv and scTv antigen recognition. To examine the scFv and scTv cell lines, the antigen was also immobilized on wells to determine the role of other cell:cell interactions (e.g. CD8 and class I MHC) in the sensitivity of T cell stimulation mediated through the CARs.

Design of the intracellular signaling domains in CAR constructs is important to elicit maximal T cell activation in response to antigen. T cell activation is thought to require two distinct signals: signal 1 is mediated by the αβ TCR and associated subunits, and signal 2 is provided by costimulatory receptors on the T cell surface interacting with ligands on the surface of antigen presenting cells (APCs). I utilized single-chain constructs that contained components capable of mediating both signal 1 and signal 2. Numerous reports demonstrate T cell activity mediated by CARs that contain components of CD3ζ (signal 1) fused to extracellular antigen receptors that mediate effector function independent of the full TCR signaling machinery (261).

Fusion of a costimulatory domain or multiple domains (signal 2) to CD3ζ could also improve antigen specific T cell activity in CAR constructs (262, 263). Since a more potent cytokine release profile has been observed with the fusion of kinase domains, such as LCK, that promote T cell activation (208, 264, 265), I included a fusion construct that contained CD28, CD3ζ, and LCK.

In the second half of this chapter, I examined a more recently adopted modification of the CD28, CD3ζ, LCK intracellular signaling cassette of the m33 scTv CAR, with the goal of further improving T cell persistence and antitumor activity. Accordingly, recent human CAR systems have demonstrated that inclusion of the 4-1BB costimulatory domain within the intracellular signaling domains of the CAR promoted T cell persistence, even 6 months after transfer (266, 267). To assess the ability of 4-1BB containing constructs to mediate T cell activity, I evaluated m33 scTv constructs containing the signaling domains of the original scTv (CD28,CD3ζ, and LCK) as well as a construct consisting of CD28, 4-1BB, and CD3ζ. The transmembrane domain (Tm) was also evaluated for effects on surface expression, comparing type I murine Tm sequences derived from either CD28 or CD8 on 58-/- T cells. The results showed that the CD28 Tm domain allowed for improved transduction efficiency.

The most significant finding was that both the high-affinity scTv and the scFv constructs were capable of mediating antigen-specific activation, in the presence or absence of CD8, when class I expressing antigen presenting cells were used as stimulators. However, CD8 had a dramatic negative effect on stimulation of T cells through both the scFv and scTv receptors when the purified antigens were coated on wells. I speculate that the effect of CD8 has to do with the sequestration of the intracellular, associated protein LCK. In the case of immobilized antigens, where CD8 is not adequately recruited to the point of the T cell synaptic interaction, LCK is sequestered by CD8 and unavailable for signaling through the scTv or scFv CAR. In contrast, when the antigen presenting cell is present, class I MHC on the antigen presenting cell can interact with CD8 that is concentrated within the T cell synapse, thereby allowing enhanced signaling through the proximal scFv or scTv molecules. These results are important, as they support a role for CD8 in interactions that do not

directly involve the TCR/CD3 complex (i.e. non-cognate class I). In addition, it suggests that current approaches using CARs may need to consider whether class I deficient tumor cells might still have a reduced ability to be destroyed by the transduced T cells, due to this role for CD8.

Materials and Methods

Antigenic Glycopeptides, Antigenic Peptides, Antibodies, and Cell Lines

Peptides (SIY (SIYRYYGL), OVA (SIINFEKL)) used in activation assays were synthesized by Penn State Macromolecular Core Facility at Penn State College of Medicine (Hershey, PA). Peptides were purified by reverse-phase chromatography using a C18 column with mass confirmed by MALDI. The glycopeptide antigen (237 antigen) used for activation assays with 237 constructs was provided as a generous gift from Stephen Evans (U. of Victoria, Canada).

Antibodies used in the current study included: goat anti-mouse $F(ab')_2$ IgG AlexaFluor 647 polyclonal antibody (Molecular Probes), anti-mVβ8 (Clone F23.2), PE-conjugated anti-mouse CD8α (Clone 53-6.7, BD Pharmingen), PE-conjugated anti-mouse CD3ε (BD Pharmingen, Clone 145-2C11), anti-CD8 antibody (Clone CT-CD8α, Cedarlane Labs), and anti-K^b antibody (Clone B8.24.3). Soluble, high affinity m67 scTv specific for SIY/K^b was expressed, refolded *in vitro,* purified and labeled as described (102). Soluble SIY/K^b peptide:MHC was expressed, refolded, and incubated with SA or SA:PE to form tetramers has been described (102).

PLAT-E (Clonetech) retroviral packaging cell line was maintained in DMEM with 10% FCS, L-glutamine, penicillin and streptomycin. T2-K^b, a TAP-deficient cell line that presents exogenous peptides, tumor cell lines (Ag104, AcosM, EL4, MC57, and B16), and $58^{-/-}$ T cell hybridoma cell lines were maintained in RPMI 1640 complete medium supplemented with 10% FCS, L-glutamine, penicillin and streptomycin. All cell lines were maintained at 37 degrees C, 5% CO_2. The $58^{-/-}$ T cell line was transduced with retroviral supernatants of the scFv or scTv constructs with or without plasmids that expressed CD8α and CD8β genes, as described (Chapter 3) (153). The C57/BL6 splenocytes used as APCs in activation assays were

harvested from mice and treated with ACK lysis buffer to remove RBCs. The remaining splenocytes were resuspended in RPMI for activation assays. The AcosM cell line was derived from the Ag104 tumor line by transfection with the CosMc chaperone protein at the University of Chicago (Hans Schreiber) (254). Thus, the AcosM cell line does not express the 237 antigen. The MC57 SIY Hi, MC57 SIY Lo, and B16 SIY tumor cell lines were transduced with a plasmid containing the SIY peptide by the Schreiber Lab as has been described (139),(268),(140).

Chimeric Receptor Gene Construction

The 789 bp DNA fragment comprised of the 237 scTv was introduced into the m33 scTv CD28, CD3ζ, LCK construct (chapter 3) by AgeI and XhoI restriction sites added by PCR to the 5' and 3' ends of the scFv respectively. The plasmid containing the 237 scFv was provided by Hans Schreiber. A XhoI restriction site within the 237 coding sequence was removed by introducing a silent mutation into the protein sequence by Quikchange (Agilent Technologies) to allow for introduction of the 237 scFv into the CAR construct. The 237 scFv CD28, CD3ζ, LCK (Figure 4.1A) construct was initially designed with two separate leader sequences derived from either the Human IgKappa antibody leader (267) (Sequence: MDFQVQIFSFLLISASVIMSRG) or the murine IgG heavy chain leader (Sequence: MGWSCIILFLVATATGVHS). I chose to pursue the human IgKappa leader sequence based on preliminary experiments which demonstrated equal transduction efficiency and antigen specific cytokine release in the $58^{-/-}$ cells in comparison with the identical 237 construct with the murine IgG heavy-chain leader (data not shown).

For construction of the 4-1BB gene constructs (Figure 4.1B) the gene sequences of the CD8αHinge, CD8Tm, 4-1BB, CD3ζ or CD8αHinge, CD28Tm, 4-1BB, CD3ζ subunits were introduced into the m33 scTv CD28, CD3ζ, LCK by XhoI and MluI sites, with the 4-1BB containing cassettes replacing the existing CD8αHinge, CD28Tm, CD28, CD3ζ, LCK subunits. The genes for the 4-1BB construct were derived from the murine 4-1BB sequence, at residues homologous to the human 4-1BB intracellular subunits in preparation for CARs in clinical trials (267). The m33 full-length TCR construct has been previously described (102).

Retroviral Gene Transfer

DNA for each of the gene constructs (Figure 4.1) was isolated by Maxiprep (Qiagen kit) and packaged into retroviral particles using the PLAT-E cell line as previously described (chapter 3). In brief, $1x10^6$ 58-/- cells were transduced with retroviral supernatants for 24 hours. Three days after transduction cells were monitored for scFv or scTv surface expression with either goat anti-mouse IgG AlexaFluor647 (10 ug/mL) or anti-mVβ8 (10 ug/mL), respectively. Cells were sorted to obtain populations expressing scTv or scFv CAR for T cell activation assays.

T cell Activation Assays

For T cell activation assays. T cells were incubated with various stimuli for 24 hrs at 37 degrees C, 5% CO_2. For all assays where peptide was titrated on antigen presenting cells or T cells were exposed to plate bound antigen, 1 equivalent of T cells, $7.5x10^4$ T cells per well, was used. For experiments where the effector to target (E:T) ratios were varied, $7.5x10^4$ target cells were used. After a 24 hour incubation, T cell supernatants were harvested and IL-2 release was quantified in an ELISA format (described in chapter 3). In experiments where plate-bound anti-mVβ8.2 (Clone F23.2) was used as stimulus, antibody was coated overnight at 10 ug/mL. For B16-SIY experiments, cells were incubated with IFN-γ at 10 ng/mL in a T75 flask 24 hour prior to the assay. Primary Cell Transduction and T Cell Killing Assays

Primary T cells were isolated from C57/BL6 splenocytes and treated with ACK lysis buffer to remove RBCs. Subsequently, cells were separated into CD4 or CD8 T cell populations using magnetic beads (MACS, Miltenyi Biotec). 1×10^6 purified cells were stimulated with anti-CD3/anti-CD28 Dynabeads (Invitrogen) per manufacturer's instructions and 30 U/mL of IL-2 24 hours prior to transduction. Retroviral supernatant was prepared as described for $58^{-/-}$ cells. Primary cells were centrifuged at 1200 x g, 30 °C for 1 hour (spinfection) with full-length TCR, scTv fusion (CD28, 4-1BB, CD3ζ) or mock (no vector DNA) retroviral supernatants, placed at 37 °C, 5% CO_2 overnight followed by a second spinfection the next morning. Cells were cultured in the presence of IL-2 (20 U/mL, day 3 post-

activation) and on day 6 were incubated with ^{51}Cr labeled T2-K^b cells pulsed with either 1 uM SIY or 1 uM OVA for four hours at 37 °C. Supernatents were isolated and ^{51}Cr was quantified for each sample using a scintillation counter. Percent specific lysis was determined relative to maximal ^{51}Cr release.

Results

Expression of scFv and scTv Chimeric Antigen Receptors in $58^{-/-}$ T Cells

The scFv and scTv chimeric antigen receptors (CARs) were cloned as fusions to the extracellular portion of CD8 (CD8α hinge), the CD28 transmembrane domain, and the intracellular domains of the CD28, CD3ζ, and LCK proteins (Figure 4.1A). The genes were expressed in the MSCV vector and transduced into $58^{-/-}$ αβ negative T cells as described (Chapter 3). As recent studies demonstrated that inclusion of the 4-1BB intracellular signaling domain in CAR constructs (Figure 4.1B) improved *in vivo* T cell persistence (267),(266),(144), this domain was also tested for activity (see below).

I first sought to evaluate the surface expression of the 237 scFv CAR in $58^{-/-}$ T cells. Cell surface expression of the 237 scFv CAR was detected using a polyclonal antibody that is specific for anti-mouse IgG (Figure 4.2A). Because this reagent is polyclonal, I reasoned that it contained antibodies capable of binding to IgG epitopes within the V_L and V_H domains of the 237 scFv. Indeed this reagent stained the transduced cells, and was used to sort a positive population (Figure 4.2A). Similarly, the scTv construct containing the high-affinity TCR m33 was transduced into $58^{-/-}$ cells, and sorted with the monoclonal anti-Vβ8 antibody (Figure 4.2B). Because the anti-IgG reagent is polyclonal, and different secondary reagents were used, it was not possible to directly compare the surface levels of 237 scFv relative to the m33 scTv. Nevertheless, both transduced populations were greater than 90% positive for surface expression and could be used to assess function.

Antigen Specific Activation of scFv- and scTv-Transduced T Cells

The 237 scFv fragment targets a tumor-specific, mutated glycopeptide expressed by the murine tumor cell line Ag104 (254). AcosM is an Ag104-derived cell line that lacks the 237 epitope; it was generated by introducing the enzyme that repairs the defect in glycosylation (responsible for the 237 neo-epitope) (254). The 237 scFv CAR-positive cells were incubated at various E:T ratios with either the Ag104 tumor line or the antigen null AcosM cell line, and IL-2 release was used to assess antigen specific activation (Figure 4.2C). The 237 CAR expressing cells were active at multiple E:T ratios with Ag104 as the antigen presenting cells (target cells), whereas no activation was observed at any E:T ratio with the AcosM line as antigen presenting cells.

To compare the efficacy of an antibody-based scFv CAR to a TCR-based scTv CAR for tumor targeting, I assayed cells that express the m33 scTv construct containing identical CD28, CD3ζ, and LCK signaling domains. Because TCRs recognize MHC-restricted peptide antigens, the scTv approach provides the potential advantage of targeting tumor stroma cells that cross-present specific pepMHC complexes in the tumor microenvironment. The scTv-positive cells were active at multiple E:T ratios in the presence of excess SIY antigen, but they were not active with the null peptide OVA (Figure 4.2D). Thus the scTv and scFv CARs could mediate similar levels of antigen-specific T cell activation.

CD8 Co-Expression Has Minimal Impact on scFv CAR-Mediated Activity with Tumor Cells

CD8 is thought to sequester LCK, promoting T cell activation in the context of class I MHC restricted antigens when the CD8/LCK complex is recruited to the immunological synapse and positioned in proximity to the cognate TCR:class I pepMHC interaction (269),(270). However, for ligands that are not class I MHC, intracellular CD8 binding of LCK could reduce the level of activation due to the sequestration of LCK away from the synapse (151),(271). As the constructs described here contained the active domain of LCK, this sequestration may not impact activity (e.g. if free cellular LCK activity is not required). To assess the

impact of the coreceptor CD8 on scFv CAR activity, the CD28,CD3ζ,LCK containing constructs were introduced into the $CD8^+$ $58^{-/-}$ T cell hybridoma line. The 237 scFv CAR $CD8^-$ and $CD8^+$ expressing T cell lines were examined for surface expression of the scFv and CD8 (Figure 4.3A). High levels of CD8 were observed on the 237 scFv, $CD8^+$ line, allowing the analysis of the impact of CD8 on activity against a non-class I MHC ligand.

Transduced T cells were incubated with either the Ag104 tumor line or the AcosM (null) tumor cell line. The $CD8^+$ and $CD8^-$ 237 lines mediated similar activity in the presence of the Ag104 tumor line, although the $CD8^+$ line yielded slightly higher maximal IL-2 release at optimal E:T ratios (Figure 4.3B). Both $CD8^+$ and $CD8^-$ lines were not stimulated by the antigen-negative cell line AcosM.

CD8 Co-Expression Completely Inhibits scFv CAR-Mediated Activity with Immobilized Antigen, in the Absence of Class I MHC

While CD8 appeared to have little impact on stimulation of the 237 scFv-mediated activation, it was possible that CD8:class I MHC interactions may still be involved since the antigen presenting cells express class I MHC. To examine this possibility, the activity of the 237 scFv CAR expressing T cell lines were examined with plate-bound glycopeptide antigen (provided by Stephen Evans, U. of Victoria, Canada (259) at various concentrations to evaluate T cell activity. The $CD8^-$ 237 scFv CAR T cell line was stimulated effectively (SD_{50}=0.7 μg/mL) by plate-bound glycopeptide antigen (Figure 4.4A). Surprisingly, the $CD8^+$ 237 scFv CAR T cell line was completely inactive at any concentration of the plate-bound glycopeptides. A control T cell line that expresses the m33 scTv was also completely negative, as expected. Thus, despite effective stimulation of the $CD8^+$ scFv cell line by antigen-positive tumor cells (Figure 4.3B), the presence of CD8 completely inhibited antigen-specific activity in the absence of an antigen-presenting cell.

To verify these results, the $CD8^+$ cell line was sorted a second time with anti-mouse IgG antibodies in order to obtain a line that expressed more homogenous levels of the 237 scFv (Figure 4.4B, compared to Figure 4.3A). This line expressed the 237 scFv at similar, homogenous levels as the $CD8^-$ cell line (Figure 4.4B). The

$CD8^+$ 237 scFv-sorted line was also inactive in the presence of glycopeptide antigen (Figure 4.4C), although it was active in the presence of the Ag104 cell line (data not shown). To test the hypothesis that CD8 contributed to T cell recognition of the Ag104 tumor line, Ag104 cells were incubated with 237 $CD8^+$ cells in the presence of an anti-CD8 antibody (clone CT-CD8α). We and others have shown previously that this antibody can inhibit T cell activation, presumably by blocking the association of CD8 and the TCR complex (151),(272),(273). Incubation with the anti-CD8 antibody at 10 μg/ml led to reduced IL-2 release by the $CD8^+$ T cell line (Figure 4.4D). This result suggests that full cell-mediated activation required association of CD8 and the 237 scFv CAR-containing the CD3ζ domains. Incomplete inhibition may indicate that some CD8 molecules could act at a greater distance from the 237 scFv CAR, through interactions with class I MHC at the synapse.

Similar Effects of CD8 on Activation Mediated by scFv and scTv CAR

Similar experiments were performed with the m33 scTv in $CD8^-$ and $CD8^+$ cells to assess coreceptor function in cells where a class I pepMHC is the ligand. However, in contrast to the normal TCR, the Cα, Cβ, or CD3 subunits are not involved in the function of the scTv, and thus CD8 may or may not be capable of associating directly with the scTv CAR upon binding to class I MHC. Cell surface levels of scTv were monitored by flow cytometry with anti-mVβ8 (Figure 4.5A). Activation of $CD8^+$ m33 scTv CAR expressing cells with peptide-loaded antigen presenting cells resulted in slightly decreased peptide sensitivity relative to $CD8^-$ m33 scTv T cells (Figure 4.5B). By this measure, the CD8 coreceptor did not appear to contribute to scTv-mediated recognition of the same class I MHC-restricted antigen. This reduction in sensitivity may be a result of sequestration of LCK, perhaps combined with the inability of CD8 to associate with the m33 scTv CAR.

To further evaluate the ability of $CD8^+$ m33 scTv cells to mediate cytokine release with plate bound antigen, m33 scTv cells with or without CD8 coreceptor were incubated with plate-bound SIY peptide:MHC tetramer. The $CD8^+$ cells were only active at the highest antigen concentrations (Figure 4.5C). In contrast, the m33 scTv $CD8^-$ cell line responded to immobilized peptide-MHC at lower concentrations,

and the maximal release of IL-2 was greater. The lower activation induced by plate bound pepMHC (Figure 4.5B) compared to cell-associated pepMHC (Figure 4.5B), is similar to observations with the 237 scFv system. However, in the m33 scTv system, the plate bound antigen is a class I MHC ligand, which in principle would allow CD8 to bind to the same molecule as the scTv. If this were the case (i.e. the m33 scTv and CD8 bound to the same molecules), one would expect enhanced signaling, as occurs with the synergistic acton of TCR and CD8 in normal T cell responses (274)**,**(215). Thus, the results shown here are consistent with the interpretation that m33 scTv and CD8 are unable to bind to the same MHC molecule. Accordingly, enhanced activation by cells that express pepMHC, compared to immobilized pepMHC, may be due to interactions between CD8 and class I MHC molecules that are proximal to the scTv:SIY/K^b interaction. Collectively, the scFv and scTv results suggest that interactions with their cognate antigens may be necessary, but not sufficient, for activation.

Effect of Antigen Density on T cell Activity Mediated by scTv and Full-Length TCRs

The cell line used for stimulation of the m33 scTv was the TAP-deficient line T2-K^b, which does not express endogenous peptides bound to K^b. These endogenous pep-K^b complexes could in principle provide synergistic responses if they are recognized as co-agonists by m33, or if they were bound at the synapse by CD8. However, the endogenous peptides also potentially decrease the density of specific antigenic pepMHC (SIY-K^b, in the case of m33), possibly affecting sensitivity of the T cells. To examine these possibilities, the activity of cells that express the high affinity $CD8^-$ m33 scTv CAR was first compared to cells that express the standard m33 $CD8^-$ full-length TCR. The CD8-negative lines were examined as CD8 did not appear to contribute to an increase in sensitivity using the T2-K^b antigen presenting cells (Figure 4.5).

Cells that express the m33 scTv (Figure 4.6A) or full-length TCR (Figure 4.6B) were incubated with antigen presenting cells that express endogenous peptides (EL4, C57/BL6 splenocytes, or MC57) or with T2-K^b which expresses primarily the exogenous SIY antigenic peptide only. In the presence of endogenous peptides, both

the scTv (28,ζ,LCK) and the conventional full length TCR exhibited decreased sensitivity as judged by the responses to EL4, C57/BL6 splenocytes, and MC57 (Figure 4.6). In order to determine if this reduction in sensitivity could be attributed exclusively to lower levels of the cognate antigen (SIY/K^b), I used a high-affinity soluble TCR (m67) to quantitatively determine the relative levels of SIY/K^b on T2-K^b compared to EL4 and MC57. In both cases, the decrease in activity was associated with lower levels of specific antigen (data not shown), no doubt due to the competition for K^b binding by the endogenous peptides. The decrease in sensitivity for the the scTv CAR was approximately 10-fold greater for the scTv relative to the full-length TCR based on the sensitization does yielding 50% maximal signal (SD_{50}). Although the basis for this is not clear, it is possible that the scTv form may rely more heavily on clustering with a sufficient density of antigenic peptide-MHC for signaling.

Transmembrane and Intracellular Domains Influence scTv CAR Transduction Efficiency and Surface Levels

Many studies have shown that different transmembrane regions or intracellular domains can impact CAR expression and activity. For example, some investigators have used the transmembrane domain from the CD28 molecule (as used in the constructs described above), while others have used the transmembrane domain from CD8 ((142)). In addition, it has been shown that the 4-1BB domain, derived from the co-stimulator molecule, provided primary T cells that express a scFv CAR with increased persistence *in vivo* in humanized murine adoptive transfer models (266), (267). The ability of a CAR to mediate T cell survival and proliferation will be important in the adoptive T cell setting. In addition, the m33 scTv-CD28,CD3ζ,LCK construct proved problematic for transduction of primary murine T cells, possibly because of the size of the DNA construct (communication with Boris Engels, U. of Chicago)(e.g. the LCK gene is 1424 bp, which may result in inefficient packaging into retroviral particles). As the 4-1BB gene is approximately ten times smaller (147 bp), it may allow for more efficient packaging and T cell transduction.

Initially, I compared the transduction efficiency of m33 scTv constructs that contained either the CD28,CD3ζ,LCK or the CD28,4-1BB,CD3ζ fusions, each with the CD28 transmembrane domain (Figure 4.1). The transductions were monitored with an antibody to mVβ8 (Figure 4.7A). The construct containing the 4-1BB domain showed a higher T cell transduction efficiency, 92.9% of cells transduced compared to the LCK containing fusion (28.7%). The trend was consistent in two separate transductions with the 4-1BB construct allowing for greater than 90% transduction efficiency. This result is consistent with the difficulty encountered in transducing primary activated mouse T cells with the LCK containing construct.

To compare the impact of transmembrane domains, a construct that contained the CD28Tm and CD8Tm were examined (Figure 4.7B). The transduction efficiencies of the two constructs, each containing the 4-1BB domain, were very good. However, the surface level of the construct that contained the CD28 Tm region was almost 10-fold higher than the construct that contained the CD8 Tm region (Figure 4.7C).

Comparison of LCK and 4-1BB Fusions in Mediating T Cell Activation

The three m33 scTv CAR constructs (Figure 4.1B) were compared with full-length m33 αβ TCR for surface expression levels after enrichment of transduced cells by cell sorting (Figure 4.8A). The two scTv CAR constructs that contained the CD28Tm were expressed at 3-fold higher surface levels than the full-length TCR, as was previously observed with the CD28,CD3ζ,LCK construct (Chapter 3). As noted above, the CD8Tm containing receptor was expressed at 10-fold lower surface levels than the CD28Tm scTv constructs, in agreement with analysis of T cells prior to cell sorting (Figure 4.7C).

To directly examine the ability of the m33 scTv CARs and full-length receptors to mediate T cell activity, cells were first tested with plate-bound anti-mVβ8 antibody as the stimulatory ligand (Figure 4.8B). T cells that expressed each of the constructs were stimulated by immobilized antibody, although the m33 CD8Tm-containing construct mediated significantly reduced activation. This result is consistent with the much lower surface levels of this receptor (Figure 4.8A). To

assess antigen specific activation, the T cell lines were incubated with SIY-peptide loaded T2-K^b cells (Figure 4.8C). The m33 scTv CD28,ζ,LCK construct mediated cytokine release with sensitivity comparable to the m33 full-length TCR. However, T cells expressing the m33 scTv CD28Tm CD28,4-1BB,ζ construct were induced only at very high concentrations of peptide. This result suggests that the LCK domain may be required for proximal signaling, or perhaps that the CD28 and CD3ζ domains need to be in tandem to promote cytokine release.

Despite the weak induction of IL-2 release mediated by the m33 scTv construct that contained the 4-1BB domain in the presence of varying SIY peptide concentrations, it is possible that higher levels of activation might be achieved at an optimal ratio of effector to target (APC). Accordingly, the scTv and full-length TCR expressing cell lines were incubated with T2-K^b SIY peptide loaded target cells loaded with excess peptide. In the presence of excess peptide (1 μM SIY, no wash), the m33 scTv CD28,ζ,LCK and the m33 full-length TCR showed similar activity at low E:T ratios (Figure 4.9). Although the LCK containing construct, and full length TCR, mediated higher levels of activation, the 4-1BB construct was clearly capable of mediating significant IL-2 release at almost every ratio, and the activation was specific as judged by the inability of the null peptide OVA to induce release.

In another set of experiments, I examined whether tumor cell lines that express the SIY peptide as an endogenous processed antigen were capable of stimulating T cells that express the various scTv and full-length TCR contructs. In the first system, the murine melanoma line B16 that expresses the endogenous peptide SIY and K^b was used (275). B16 expresses low levels of MHC K^b but incubation with IFN-γ results in upregulation of MHC and antigen presentation. Incubation of B16/SIY with IFN-γ resulted in upregulation of the MHC allele K^b and improved expression of SIY/K^b as monitored by an anti-K^b antibody and the soluble high-affinity m67 scTCR, respectively (Figure 4.10A). Incubation of the IFN-γ induced B16/SIY cell line with the various m33 scTv and full-length constructs revealed that only the LCK containing scTv and full-length TCR construct were able to release cytokine. In the case of the non-IFN-γ-induced B16/SIY, only the full-length TCR

mediated significant IL-2 release. Thus, the hierarchy of sensitivity for the three consructs was full-length TCR>scTv-LCK>scTv-4-1BB.

A similar set of experiments was carried out with the murine-derived MC57 lines that were previously transduced with a vector that expresses the SIY peptide endogenously, yielding high and low levels of SIY antigen (268). In each of these systems only the m33 full-length TCR was able to mediate cytokine release (Figure 4.10B).

Comparison of m33 scTv/4-1BB Fusion and Full-Length TCR in Mediating Cytolytic T Cell Activity

While the T cell hybridoma serves as a surrogate for examining some properties of T cells, it can not be used to study many aspects of T cell function. These include cytotoxic activity, T cell proliferation, or *in vivo* properties such as T cell persistence and proliferation. In fact, the original premise of using the 4-1BB domain involved its ability to serve a co-stimulatory function in the proliferation of T cells. Thus, it is possible that the 4-1BB containing constructs will be of sufficient potency to kill target cells and possibly to mediate proliferation when introduced into primary T cells.

To begin to address these issues, I have worked with Dr. Adam Chervin in the lab to transduce primary T cells, activated with immobilized anti-CD3/anti-CD28 antibodies (activation is required for transduction). Our results showed that about 1 to 5% of $CD8^+$ and $CD4^+$ cells were transduced with the m33 TCRs, as indicated by staining with SIY/K^b tetramer (Figure 4.11A, C). Despite this relatively low frequency of transduced cells, both the full-length TCR and the m33 scTv containing the CD28Tm and 4-1BB domains were capable of mediating efficient lysis of T2-K^b cells incubated with SIY peptide in both $CD8^+$ (Figure 4.11B) and $CD4^+$ T cells (Figure 4.11D). This result shows that the m33 scTv/4-1BB construct could be transduced into primary T cells, that the receptor could directly recognize and mediate signaling that leads to the destruction of target cells, and that not only $CD8^+$ T cells, but $CD4^+$ T cells were capable of mediating lysis. Future studies will allow

us to examine if these transduced T cells persist *in vivo*, and can undergo antigen-induced, and/or homeostatic proliferation.

Discussion

Despite numerous studies utilizing CARs against a wide array of tumor antigens (reviewed in (155)), often as fusions to CD28 and CD3ζ, the mechanism and efficiency (compared to normal TCRs) by which these receptors are able to mediate activity is not well understood. Assuming that the mechanism of signal transduction by the CAR is similar to a full-length TCR, there are two possible mechanisms of signaling; both mechanisms require that the kinase LCK phosphorylates specific intracellular tyrosine activation motifs (ITAMs) within various transmembrane proteins, including CD3ζ, that subsequently potentiate T cell activation (Reviewed in (103)). One hypothesis proposes that there is a mechanical force effect on the full-length TCR that causes conformational changes within the TCR, such that ITAMs (immune tyrosine activation motifs) on accessory CD3 proteins are made more accessible within the cell, thereby facilitating ITAM phosphorylation by the kinase LCK (106),(276). Another hypothesis suggests that the clustering of TCRs results in aggregation of LCK at the immunological synapse near CD3 subunits, resulting in T cell activation (277),(278),(279). In this work, I do not distinguish between these two models, but rather propose that some basal level of LCK interaction with CD3 subunits on CARs is necessary to drive T cell activity (108).

Chimeric antigen receptors derived from both an scFv and scTv were compared with the goal of evaluating targeting overexpressed MHC unrestricted cell surface antigens relative to overexpressed tumor associated peptide-MHC complexes respectively. Collectively, my data demonstrated that scFv and scTv CARs containing CD28, CD3ζ, and LCK intracellular subunits were able to mediate similar cytokine release when antigen was presented at high levels, as long as the scTv was engineered to have higher affinity than a wild type TCR (Figure 4.2). Experiments comparing scTv constructs that lacked LCK (4-1BB constructs) with the CD28, CD3ζ, LCK scTv showed that the CD28, 4-1BB, CD3ζ construct (lacking LCK) had

lower maximal cytokine release in response to APCs (Figures 4.8, 4.9 and 4.10). In perhaps the most unexpected aspect of the current work, I show that CD8 is important in the activity of the scFv- and scTv-CARs, presumably through interactions with the non-cognate class I molecules on the surface of the target cells. Discussions of the proposed mechanism involved in this effect, and other implications of these studies follow.

Non-CAR-Associated LCK Appears to be Required for Maximal CAR-Mediated Cytokine Release

Other studies have demonstrated that CD8 associates with intracellular LCK, decreasing the pool of 'free' (noncoreceptor-associated) LCK available to localize with cell surface proteins (151), (271). In the context of full-length TCR, CD8-associated LCK is brought into proximity with the TCR/CD3 complex, and thus CD8 acts as a coreceptor, improving the T cell sensitivity to antigen (61). However, in these cases, the same cognate class I pepMHC molecule recognized by the TCR could be bound by CD8, thereby "delivering" LCK to the site of its substrates (e.g. CD3ζ). I sought to determine if CD8 could also play a similar role with scTv and scFv constructs, even though in the latter case the cognate antigen is not a class I ligand and thus not capable of direct binding by CD8**.**

Based on my results, the chimeric antigen receptors that contained CD28, CD3ζ, and even the LCK domain appeared to still require the LCK associated with CD8 to allow productive signaling and cytokine release. Most strikingkly, this interpretaion is supported by the finding that 237 $CD8^+$ CAR constructs showed no detectable activity in response to plate-bound antigen, whereas the same cell line was stimulated very efficiently by target cells that expressed the antigen, and class I MHC. Conversely, the same construct was stimulated by plate bound antigen when expressed in a CD8-negative T cell (Figure 4.4A, 4.4C). The most straight forward interpretation of these results is that CD8 could not participate in recognition of the plate bound glycopeptide antigen because MHC was absent, and in this case, the requisite LCK was sequestered away from the scFv-CAR at the point of contact between scFv and antigen (i.e. the immunological synapse). Similarly, $CD8^+$ m33

scTv could have been much less active with plate-bound pepMHC because CD8 could not interact with the same antigenic pepMHC molecule as the scTv. In both instances, LCK sequestration away from the scTv or scFv by CD8 prevented or decreased T cell cytokine release.

In the presence of antigen presenting cells (APCs), $CD8^+$ 237 scFv (Figure 4.3B, blue line) and $CD8^+$ m33 scTv (Figure 4.5B, blue line) could mediate efficient cytokine release, presumably because CD8 interacted with MHC molecules on the APCs, thus recruiting LCK to the synapse at the TCR:APC interface. However, the CAR-associated LCK appeared to also be important for cytokine release as shown for experiments where LCK was not part of the CAR-fusion, as the CD28, 4-1BB, CD3ζ m33 scTv construct (Figure 4.8). Thus, my experiments suggest that CAR-fused LCK and the CD8-associated LCK were both important for maximal CAR-mediated T cell activity.

These results suggest that the fused LCK domain is not by itself sufficient for mediating activity. One possible explanation involves the partitioning of cell surface receptors into lipid rafts, which are less accessible to regulators of proteins involved in proximal T cell signaling events. A bulky transmembrane protein phosphatase, called CD45, regulates LCK function by de-phosphorylation of a tyrosine; CD45 is not incorporated into cholesterol rich lipid rafts where TCR and associated coreceptors often exist (109),(110). Hence, it is possible that the observed requirement for non-CAR-associated LCK is due to the inefficiency by which LCK on CAR fusions is activated because of its reduced accessibility to CD45. This model is complicated by the fact that LCK has two regulatory phosphorylated residues, at positions Tyr394 and Tyr505; these need to be phosphyorylated and dephosphorylated respectively to initiate LCK activity (280). Lack of CD45 within lipid rafts prevents removal of the phosphate at Tyr394, allowing activation of LCK. Tyr505 is dephosphorylated, upon T cell signaling, by the kinase Csk which is associated with rafts. In contrast, association of CD45 and Tyr394 of LCK results in removal of phosphate at this tyrosine and loss of kinase activity (281). Thus, CD45 has both a stimulatory and regulatory role for T cell function which may modulate LCK in CAR activity (282). Future studies will be required to evaluate the relative

localization of CD45 to scTv and scFv CARs (e.g. by fluorescence resonance energy transfer (FRET) or immunoprecipitation (IP) pull-downs/western blots) and the phosphorylation status of the CAR-associated LCK versus the cellular LCK under different activation conditions.

A Model for the Contribution of CD8 to scFv CAR Activity

Previous studies have shown that the addition of tyrosine kinases such as LCK to CAR constructs improved maximal cytokine release (264),(283), as my results imply in comparison of the LCK and 4-1BB constructs (Figure 4.8). However, the previous CAR studies did not assess the contribution of CD8 in mediating T cell activity since most CARs evaluated to date recognize antigens unassociated with MHC proteins. I used the 237 scFv-fusion system to examine this issue, comparing the responses to plate-bound antigen (without class I MHC) or tumor cells (with class I MHC). As described above, the $CD8^+$ 237 scFv T cells were inactive with plate-bound glycopeptide antigen (Figure 4.4A, 4.4C) but mediated cytokine release in the presence of the Ag104 tumor cell line that expresses 237 antigen and MHC molecules (Figure 4.3B). I propose that this difference in response is based on the CD8 recognition of MHC molecules that are in close proximity to antigenic 237 epitopes on the tumor cell lines; the proximity allows CD8-associated LCK to be brought into proximity with the 237 scFv CAR, initiating T cell signaling. A similar role for the CD4 coreceptor has been observed with full-length TCR by FRET, whereby CD4 can bind to MHC molecules that neighbor the TCR:pepMHC interaction and contribute to initiation of T cell signaling (284). However, to my knowledge, this productive signaling interaction between CD8 and non-cognate class I MHC has not been revealed in previous studies.

Further, I propose a model where in the absence of CD8 coreceptor, signaling through the scFv and scTv CAR molecules is facilitated by the activity of non-CD8-associated cellular LCK (called “free” LCK in some studies), which initiates T cell activation upon antigen binding (Figure 4.12A). In the presence of CD8, however, LCK sequesters CD8 away from the scTv CAR complex (Figure 4.12B). In the case of the m33 scTv, the CAR cannot act cooperatively with CD8 to recognize the same

pepMHC antigen as the scTv, and CD8 also sequesters LCK away from the immunological synapse, effectively decreasing T cell sensitivity to antigen (Figure 4.5B). Alternatively, LCK could be brought into close proximity of the scTv-CAR through CD8 binding to MHC molecules that neighbor the pepMHC class I antigen bound by the scTv (Figure 4.12C).

Partial inhibition of the activity of 237 scFv CAR in $CD8^+$ T cells with an anti-CD8 antibody (Figure 4.4B) is consistent with this model. The incomplete inhibition could be due to several factors. The anti-CD8 antibody is thought to bind at a site that does not prevent interaction with class I MHC, but interferes with interactions with the normal TCR complex. Thus, the antibody-bound CD8 could act at a longer distance to interact with scFv (relative to scTv) through recognition of 237 antigen epitopes in different orientations from the membrane, or by *trans* activation of two neighboring scFv CAR molecules (i.e. LCK from the neighboring scFv phosphorylating the ITAMS of other nearby scFv molecules). Regardless of the explanation, my experiments support a model where CD8 contributes to target cell recognition and signaling even in cases where the CAR recognizes antigens that are not MHC proteins.

Implications of the Role of CD8 on scFv-CAR-Mediated Adoptive T Cell Therapies

A role for CD8 participation in tumor cell recognition with scFv receptors targeting non-MHC antigens has important implications for tumor immunotherapy. Many tumor cells downregulate MHC molecules to evade the immune response, creating 'hard lesions' within tumors that often evade T cell responses (reviewed in (285)). In these instances scFv directed at MHC unrestricted antigens would be unable to mediate maximal T cell response to tumor, because CD8 could not contribute to T cell:APC interaction (Figure 4.12D). This mechanism has important implications for CARs directed against non-MHC antigens, since CAR constructs are often transduced into $CD8^+$ T cells to destroy cancer cells and MHC loss on tumor cells would potentially decrease the efficacy of CD8+ T cell activity.

The lack of cooperativity between CD8 and scTv in antigen recognition, as opposed to the cooperative effects of CD8 with full-length TCR is interesting in that

it is not well understood if the CD8 coreceptor associates with the extracellular domains of the TCR to provide enhancement or if the MHC mediates CD8/TCR surface aggregation (286),(287) . The scTv system provides an opportunity to probe CD8 association with TCR in antigen binding by comparing the role of CD8 in scTv and full-length TCR. An additional unresolved issue with CAR constructs is the mechanism of CD28 costimulation. One hypothesis suggests that CD28 in the CAR transmembrane domain permits heterodimerization with other CD28 subunits allowing for costimulatory signal from CD28 ligands on target cells, but has yet to be explained (288). Thus, a multitude of issues regarding TCR and CAR assembly, mechanism of action, and function could potentially be studied in the $58^{-/-}$ system with the m33 scTv and 237 scFv in future experiments.

Modification of Intracellular Signaling Domains to Include 4-1BB For Improved CAR Expressing T cell Persistence

A major challenge in adoptive therapy is assuring that T cells expressing CAR constructs persist, to eliminate and prevent outgrowth of tumor. To provide maximal activity of the scTv constructs, I explored the use of 4-1BB signaling for improved persistence of transduced T cells. Although the 4-1BB constructs are transduced with high efficiency, they did not mediate cytokine release with similar maximal IL-2 release or antigen sensitivity in comparison to the CD28, CD3ζ, LCK construct *in vitro*. Two possible explanations for the decreased antigen sensitivity of the 4-1BB constructs are that 1) the CD28 and CD3ζ constructs are ineffective when separated by the 4-1BB domain in *cis* for mediating cytokine release or 2) that the murine homologue of 4-1BB is not functional. Modifications of the intracellular signaling domains may improve T cell cytokine release, either by swapping of domain orientation or inclusion of other signaling domains. It will be important to assess the potency of other intracellular signaling domains, namely receptors containing only CD3ζ Tm and intracellular CD3ζ, as recent work has shown these receptors provide maximal T cell activity (289). It is unclear if T cells expressing CARS containing the CD3ζ Tm would persist, as another study has shown that receptors containing this transmembrane domain fail to persist *in vivo* in murine models (267). Another

possibility would be to express two CARs of differing specificity in the same retroviral vector with different signaling subunits. For instance, other groups have introduced two CARs with different signaling subunits (i.e. CD3ζ in one CAR and CD28 in another CAR) to allow for maximal signal 1 (CD3ζ membrane proximal) and maximal signal 2 (CD28 membrane proximal) (290).

Although preliminary data with the murine tumor lines overexpressing SIY suggested that the 4-1BB and LCK containing scTv were inefficient in mediating cytokine release (Figure 4.10), it is possible that the costimulatory domains present in these constructs allow for targeting by T cells in differing states of activation (291). In point of fact, preliminary results with the m33 scTv 4-1BB construct introduced into primary T cells have demonstrated that the transduced cells were able to lyse SIY-expressing target cells (Figure 4.11, experiment performed by Dr. Adam Chervin). Thus, the costimulatory domains may be sufficient to drive T cell killing of target cells and this response may be sufficient to eliminate tumor burden if transduced T cells can persist *in vivo*. Improvements in transduction of primary murine T cells, particularly with the smaller 4-1BB containing constructs, will allow for comparison of the 237 scFv with the m33 scTv for targeting tumors in adoptive transfer formats. It should be noted that the lysis of SIY/K^b+ target cells by scTv and scFv transduced primary T cells was quite potent, especially given the low levels of expression currently obtainable (< 10% in most cases) in primary cells.

To date, there are no reports to my knowledge using the murine homologue of 4-1BB in chimeric antigen receptor formats. It is important that the murine versions of these proteins be evaluated *in vivo* in murine models, as the humanized versions of two CARs specific for tumor antigens led to adverse events, which potentially could have been avoided by evaluation of a murine model system (292), (293). In one of these instances, it is hypothesized that low levels of target antigen were present on non-malignant tissue, which caused autoimmunity from CAR transduced T cells targeting self-tissue. This led to systemic inflammation and multi-organ failure (292). Thus, there is a clear need to explore murine model systems with CARs that express antibodies targeting murine antigens to understand reactivity with self tissue, as many of the preclinical studies are performed in humanized murine systems where target

antigen expression is limited to a xenograft tumor. Such model systems often fail to mimic the effect of low levels of self-antigen on non-malignant tissue. This is less of a concern for the 237 antigen, since expression is thought to be limited to tumor tissue. From the existing murine studies evaluating scFv-derived CARs, it is apparent that the T cell mediated response is much more potent than the response mediated by the soluble antibody (294), and future work will need to evaluate the appropriate conditions for adoptive transfer of CAR expressing T cells which could be evaluated with the 237 and m33 CAR constructs (i.e. choice of antigen target, or number of transduced T cells to introduce to patients).

Antigen Density Effects on scTv and scFv Function

In the context of the tumor environment the amount of antigenic peptide:MHC is lower in surface density than in the context of SIY loaded T2-K^b cells that only express one antigenic peptide (data from SIY/K^b system not shown). I observed decreased antigen sensitivity of the m33 scTv and m33 full-length construct using antigen presenting cells which present other endogenous peptides. Quantification of the surface levels of antigen demonstrated that the decrease in sensitivity was due to lower levels of antigen in the cell lines expressing endogenous peptides. The decrease in sensitivity was larger for the scTv relative to the full-length TCR, further implying that the signaling of the CAR constructs might rely on clustering to mediate activity.

Others have demonstrated that scFv CARs require 10,000 - 15,000 antigen epitopes/target cell for half-maximal lysis, with ~1,000 epitopes required for T cell activation (as observed by TCR downmodulation) (295). This number is much larger than the number of antigens recognized by conventional full-length αβ TCRs as it is estimated that with $CD8^+$ full-length TCR can mediate activity with as few as 10 peptide:MHC molecules (296),(297). In the context of cancer cells, the number of specific pepMHC are likely lower than the number of surface molecules targeted by scFv therapies, and hence, it will be important to find ways to improve scTv CAR function by either modification of the intracellular signaling domains or by developing fusion proteins that allow CD8 coreceptor to aid in scTv target cell

recognition by modification of the hinge region. Work is on-going to determine the number of antigenic molecules present on Ag104 cells and the various SIY/K^b expressing tumor cell lines to draw quantitative comparisons and determine the parameters for scFv and scTv activation.

Because CD8 is a fixed length from the T cell membrane, some antigenic pepMHC epitopes may not be in close enough proximity to MHC molecules to allow for CD8 coadhesion at the T cell:APC interface. For instance, the m33 scTv recognizes peptide-MHC antigen at a fixed length and orientation from the membrane and steric effects could possibly limit the ability of CD8 to bind to the same or neighboring MHC complexes as the scTv (i.e. Figure 4.12B). The length of the CD8α hinge spacer connecting the scTv to the T cell may also be too long or short to accommodate binding by CD8 to the same MHC as the scTv fusion. In contrast, the 237 scFv has epitopes at varied distances from the cell membrane which may allow for CD8 coadhesion to neighboring MHC molecules spanning longer distances across the membrane. I speculate that variation in the hinge length may potentially afford advantages in creating CD8 cooperativity for scTv constructs at yet to be defined distances from the APC membrane.

The Effect of Hinge Region Length Between scFv or scTv and Tm Domain

Others have demonstrated with different scFv the importance of CAR hinge region for improving CAR activity depending on the length if antigen from the APC membrane. Epitopes that were in close proximity to the target cell membrane were targeted more effectively (295),(298). Another study using BiTe molecules (BiTe: bispecific T cell engager), which consist of antibodies with similar affinity for antigen fused to scFv anti-CD3 antibodies, also showed that epitopes closer to the target cell membrane were efficiently targeted (299). Notably, the antibodies used in this study were of comparable affinity for antigen, suggesting that the primary determinant of BiTe molecule efficacy was target antigen distance from the APC membrane. In full-length TCR systems increasing the length of peptide:MHC from target cell membrane caused less potent T cell activation, consistent with a model where TCR-ligand pairs are large and CD45 infiltration near the TCR negatively regulates T cell signaling

(300). Yet other studies state that the length of the hinge region can compensate for distance from the target cell, with longer hinge regions for some antigens promoting improved T cell activity (301). These studies all suggest that for each antigen, there may be an optimal length for CAR interaction, and potentially a specific length of antigen:receptor interaction that would allow CD8 coreceptor association. Specifically, in the context of the m33 scTv, an appropriate sized linker may allow for CD8 cooperativity improve scTv sensitivity, such that fewer antigenic pepMHC molecules could be recognized. A thorough analysis of the effect of spacer region or Tm domain has not been undertaken and is needed, as few groups have evaluated scFv CAR hinge length in regards to antigen choice.

The importance of the hinge length in CAR function is highlighted by the multitude of spacer regions used in various different scFv-based constructs. Given that different spacer regions can improve CAR function and could potentially allow for CD8 cooperativity with the scTv, a selection strategy to determine the optimal hinge region would be valuable. Our lab has previously developed a selection strategy for introduction of TCRs into T cells and isolation of receptors with improved biological properties (147). One potential strategy to determine the optimal spacer region for a given antigen would be creation of a library of the 10-20 most prevelent spacer regions in a CAR format derived from human extracellular cell surface proteins. ScFvs of a wide variety of specificities could be introduced into a template CAR and subsequently, libraries containing spacer regions of different lengths could be generated. A similar strategy could be used with the m33 scTv CAR, to attempt to isolate hinge regions that might permit CD8 cooperativity. An *in vitro* (IL-2 release assay) or *in vivo* (a mouse containing antigen presenting tumor cells) selection strategy could then be used to isolate the cells which mediate the greatest level of cytokine release or tumor infiltration. Such a strategy would be extremely valuable to the field, as scFv could be evaluated for the optimal spacer without the need to evaluate each spacer region individually, a time consuming process for every antigen. More importantly, a hinge region that promoted CD8 cooperativity with an scTv could allow for recognition of low numbers of pepMHC molecules and mimic full-length TCR recognition in the presence of CD8.

CD28Tm Domain Provides an Advantage for Improved Surface Expression Levels in Murine T cells

I also compared the murine CD8Tm and CD28Tm domains, as many CAR constructs use different transmembrane domains derived from various cell surface proteins (reviewed in (142)). CD8Tm was chosen for evaluation as recent clinical trials used receptors with an identical CD8Tm for constructs targeting CD19 for B cell malignancy (302), (303) and HER2 for metastatic melanoma (267). The CD8Tm conferred a marked reduction in surface levels of scTv relative to the CD28Tm. Importantly, the CD8Tm sequence is identical in mouse and man, suggesting that there may be some issues associated with using this sequence in human CARs. The CD8Tm is shorter in length than the CD28Tm domain, but has a very similar hydrophobicity index (data not shown), suggesting that the improved surface levels of the CD28Tm constructs are due to undefined characteristics of this transmembrane protein sequence. Others have shown that the CD8α transmembrane domain promotes homodimerization with other CD8α subunits, but the transduced $58^{-/-}$ cells lacked CD8, so it is possible that lower surface levels were due to a lack of endogenous CD8 that would permit heterodimer formation with introduced CAR. It is also possible that CD28 is able to dimerize with endogenous CD28, enhancing CAR surface levels of the CD28Tm.

The CD28Tm is not as highly conserved between murine and human proteins, suggesting that the enhanced surface levels obtained in the CD28Tm system will not necessarily provide benefit in human systems. As many CARs directed at human antigens use the CD28Tm, using this Tm is beneficial because it is the closest Tm mimic to analogous human receptors. A selection strategy similar to that described for identifying optimal CAR hinge regions could be used to select the most advantageous transmembrane domain to improve CAR surface levels, and determine which transmembrane domains permit that most efficient surface expression while retaining CAR signaling capabili

Figures

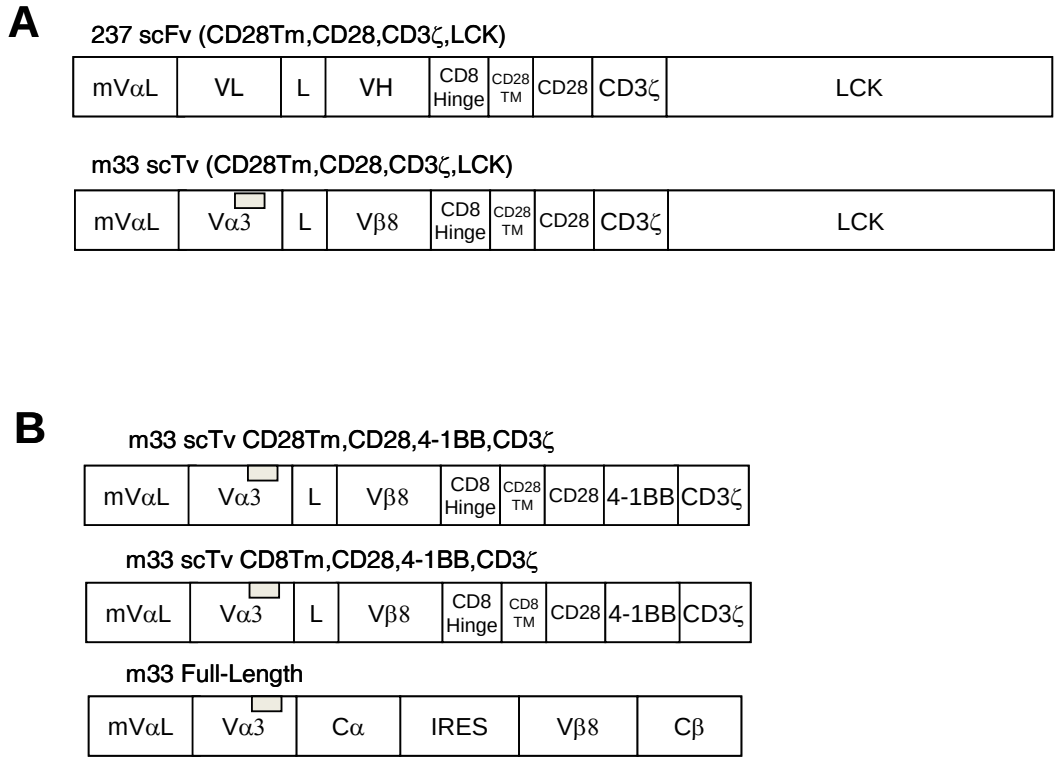

Figure 4.1. Schematic of 237 scFv and m33 scTv CAR Constructs.
Tm=Transmembrane Domain. mVαL= murine Vα Leader sequence from 2C TCR. The gray box represents the mutations which confer enhanced affinity of the m33 mutant of the 2C TCR that improves affinity for SIY/K^b.

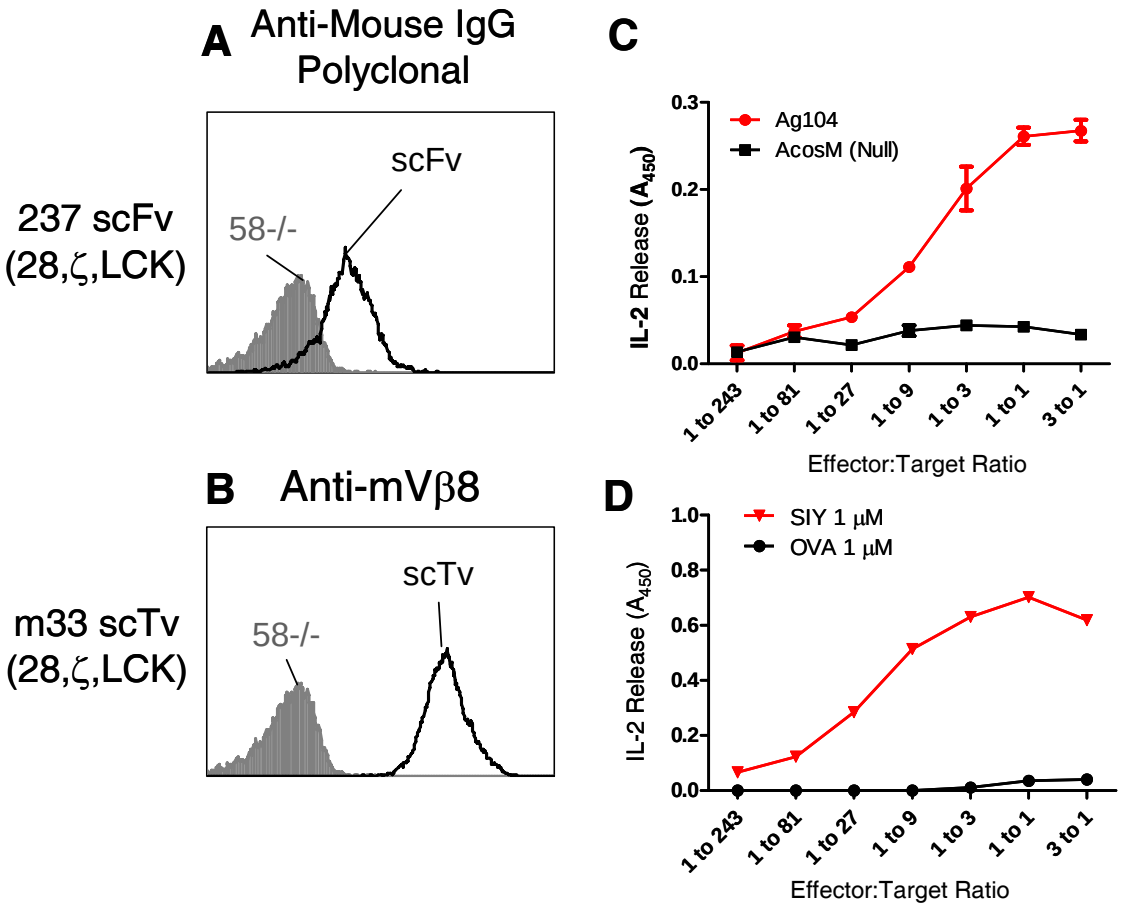

Figure 4.2. Surface Expression and Activation with Tumor or Peptide Loaded Antigen Presenting Cells. **(A)** Surface expression of the 237 scFv CAR (black line) or $58^{-/-}$ $CD8^-$ T cell line (gray). **(B)** Surface expression of the m33 scTv CAR (black line) or $58^{-/-}$ $CD8^-$ T cell line (gray). **(C)** Activation of the 237 scFv line from (A) with Ag104 (red, antigen +) or AcosM (black, antigen null) tumor cells. Data is representative of 3 independent experiments. **(D)** Activation of m33 scTv line from (B) with T2-K^b cells pulsed with SIY (red) or OVA (black) peptide. Data is representative 2 independent experiments.

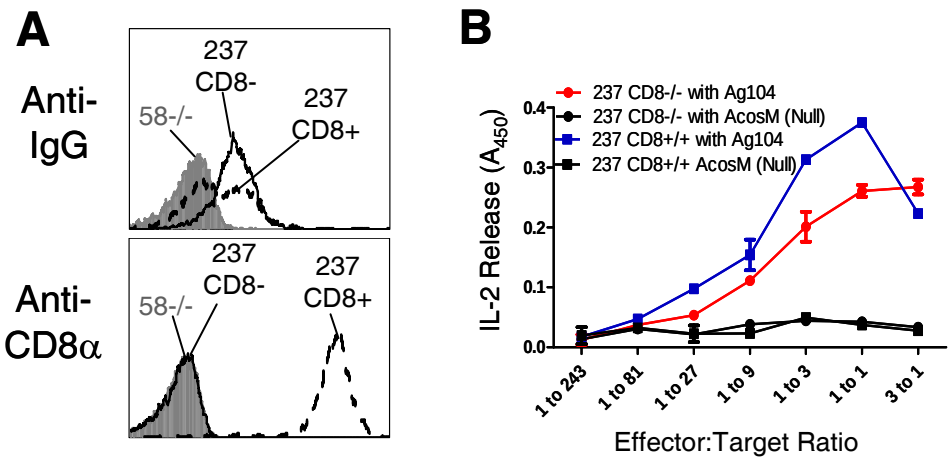

Figure 4.3. Activation of CD8$^-$ and CD8$^+$ 237 scFv CAR Expressing T cells with Tumor Cell Lines. **(A)** Surface expression of 237 scFv and CD8α with 58$^{-/-}$ (gray, filled histogram), 237 CD8$^-$ (black solid line), and 237 CD8$^+$ (black dashed line) cell lines as indicated. **(B)** CD8$^+$ (squares) cells from (A) were stimulated with Ag104 (blue) or AcosM (null, black). CD8$^-$ cells (circles) from (A) were also stimulated with Ag104 (red) or AcosM (null, black). Data from B is representative of three independent experiments.

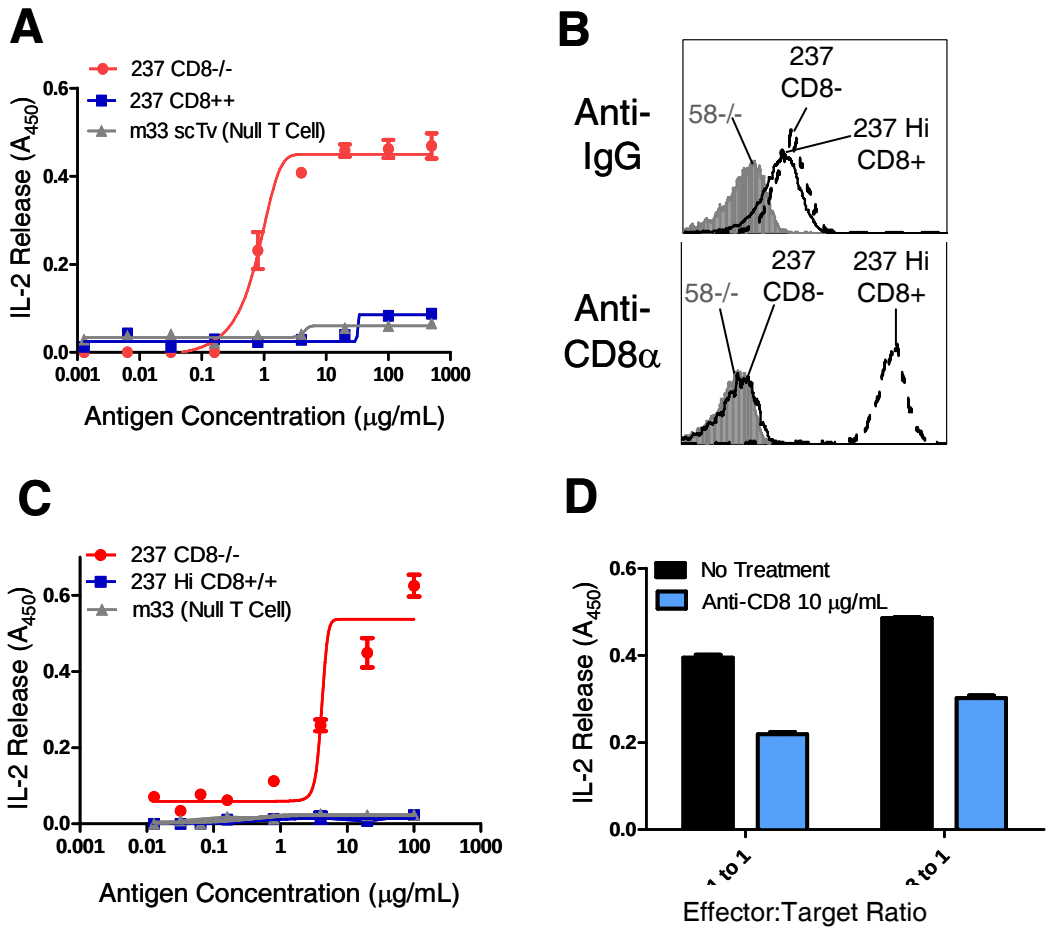

Figure 4.4. Activation of CD8$^-$ and CD8$^+$ 237 scFv CAR Expressing T cells with Plate-Bound Glycopeptide Antigen and Effect on T cell Activation from Blocking CD8:CAR Association. (A) Plate-bound glycopeptide activation of 237 CD8$^-$ scFv (red), 237 CD8$^+$ scFv (blue), or m33 CD8$^-$ scTv (null T cell, gray). **(B)** Surface expression and CD8 expression of 237 HI CD8$^+$ expressing line after sorting for homogenous CAR expression. **(C)** Plate-bound glycopeptide activation of cell lines shown in (B). Data from A and C is representative of three independent experiments. **(D)** 237 HI CD8$^+$ cell line from (B) was incubated with Ag104 cells at given E:T with (light blue) or without (black) pretreatment with anti-CD8α blocking antibody at 10 μg/mL. Data represents the mean of 4 wells for each sample.

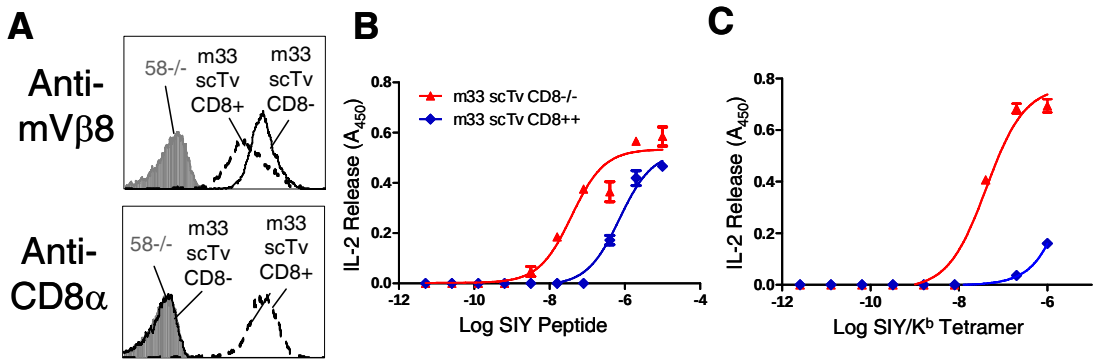

Figure 4.5. Surface Expression and Activation of m33 scTv CAR With or Without CD8 Coreceptor. (A) Surface expression and CD8α levels of m33 $CD8^-$ and $CD8^+$ scTv cell lines as indicated. **(B)** Activation of $CD8^-$ (red) or $CD8^+$ (blue) cell lines with peptide pulsed T2-K^b cells. No activity was observed with OVA pulsed cells (data not shown). Data is representative of three independent experiments. **(C)** Activation of m33 $CD8^-$ (red) or $CD8^+$ (blue) scTv cell lines from (A) with plate bound SIY/K^b tetramer. No activity was observed with OVA/K^b tetramer (data not shown). Data is representative of two independent experiments.

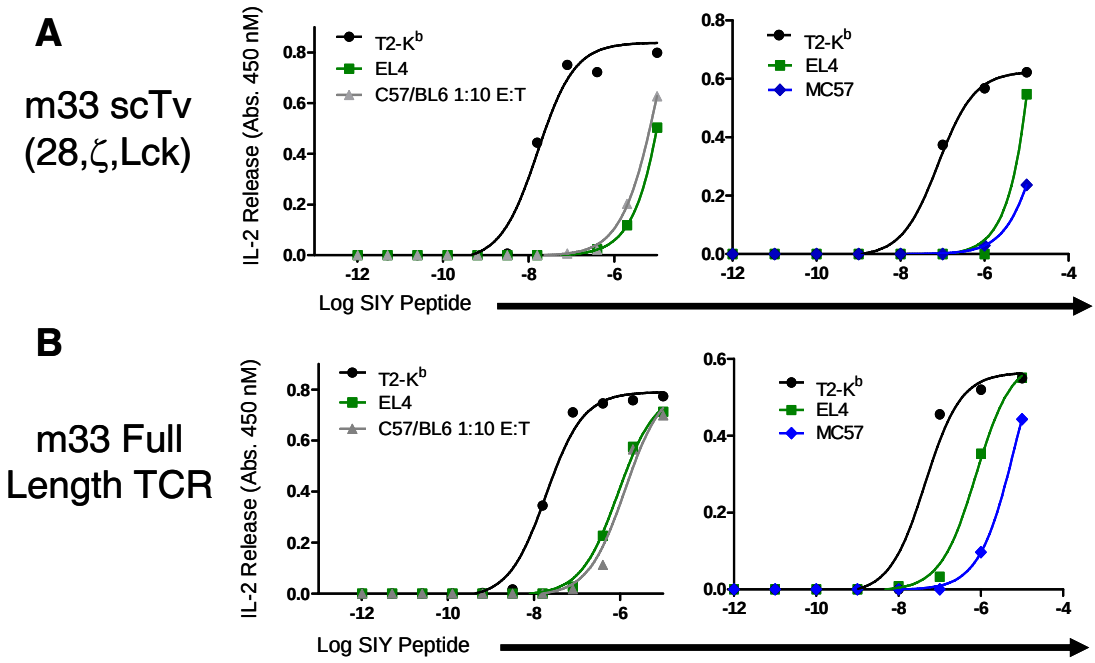

Figure 4.6. Endogenous Peptides Decrease SIY/K^b Surface Levels and Receptor Antigen Sensitivity. (A) SIY peptide was loaded onto T2-K^b (black), EL4 (green), C57/BL6 splenocytes (gray), or MC57 (blue) cells for 24 hrs with m33 CD8$^-$ scTv and IL-2 release was measured. **(B)** m33 CD8$^-$ full-length TCR was used in place of scTv with the APCs from (A).

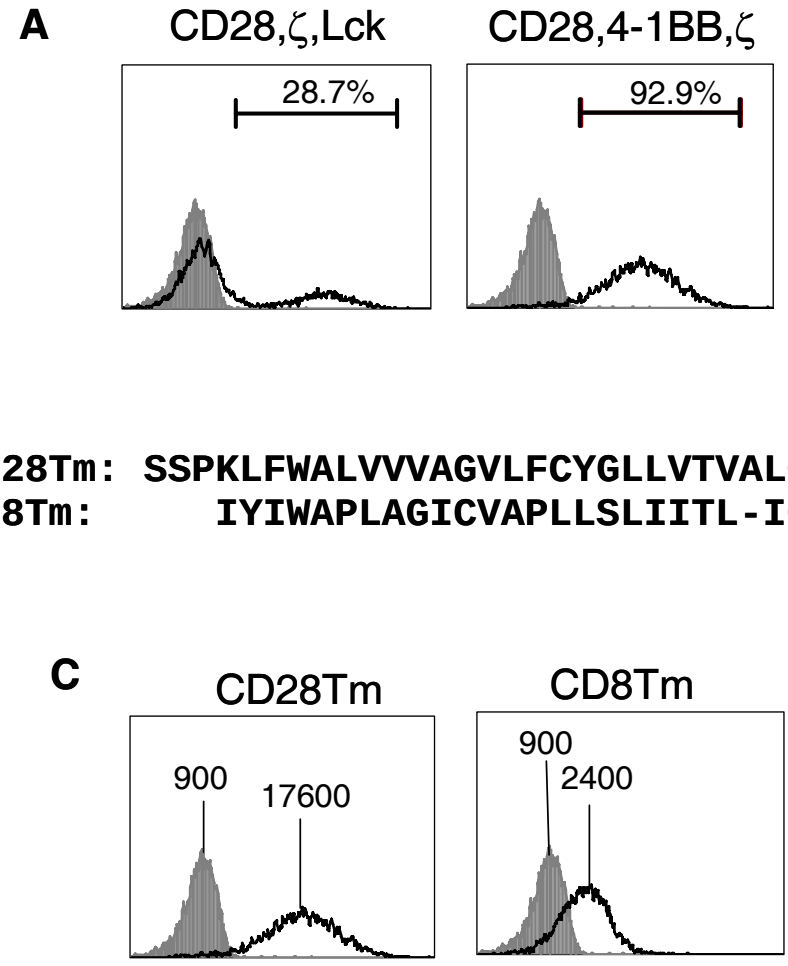

Figure 4.7. Transduction Efficiency and Initial Surface Levels of m33 scTv Constructs. (A) Surface expression of scTv containing CD28, CD3ζ, LCK or CD28, 4-1BB, CD3ζ intracellular signaling subunits (ζ= CD3ζ). Untransduced $58^{-/-}$ T cells (gray filled histograms) or transduced cells (black line) were analyzed for Vβ8 expression. Percentage represents portion of cells within marker shown for transduced cells before cell sorting. **(B)** Alignment of CD28 and CD8 murine transmembrane domains. **(C)** Comparison of CD8Tm and CD28Tm with CD28, 4-1BB, CD3ζ scTv constructs. Untransduced $58^{-/-}$ T cells (gray) or transduced cells (black line) were analyzed for Vβ8 expression as in (A). Peak mean fluorescence intensity is labeled for each histogram, and CD28Tm constructs were expressed with 7-fold higher MFI than CD8Tm constructs.

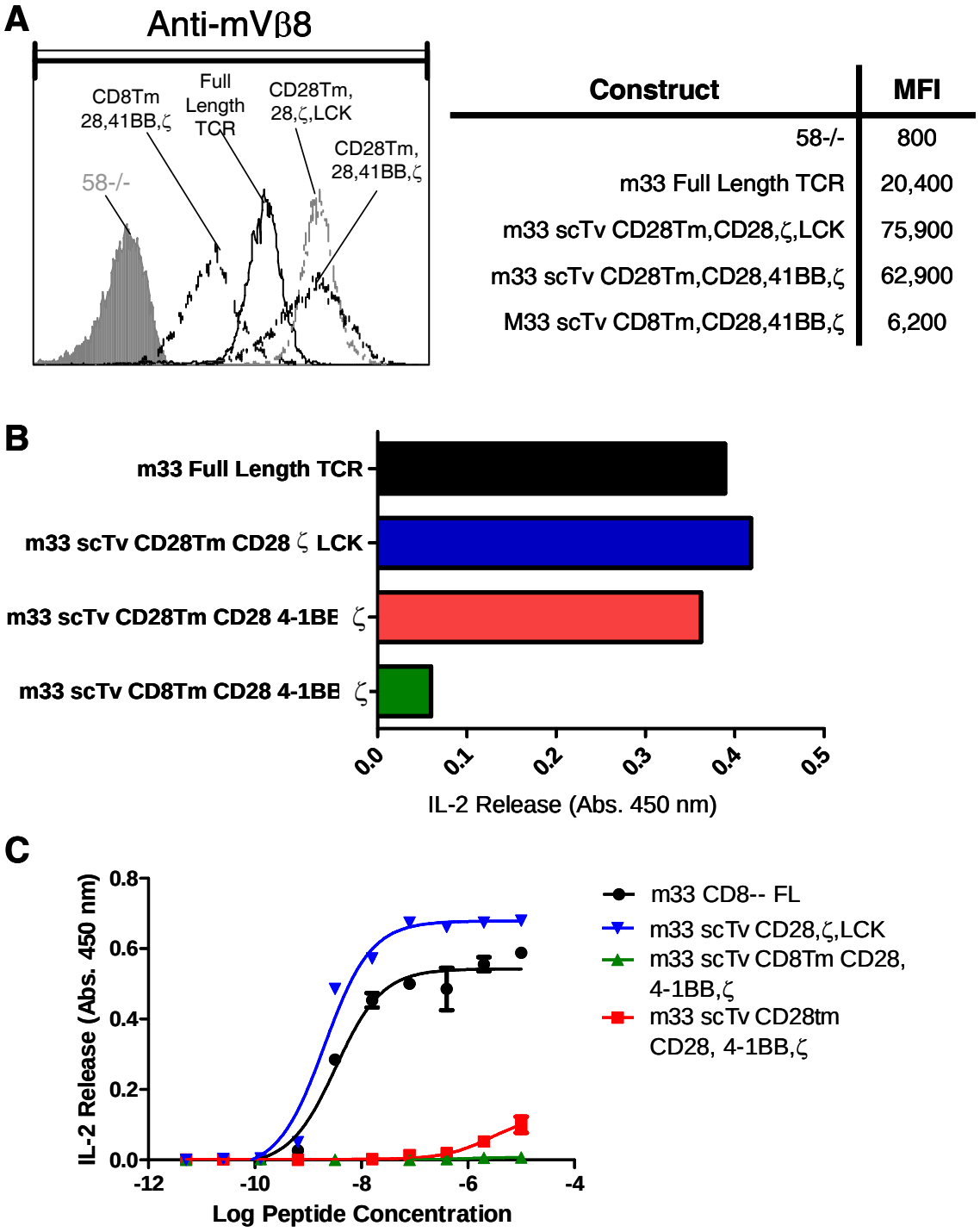

Figure 4.8. Surface Expression and Characterization of 4-1BB Containing scTv Constructs. (A) Surface levels of scTv and full-length constructs after selection. MFI=mean fluorescence intensity of the entire population of T cells. **(B)** T cell activation with plate-bound mVβ8 antibody. Data is representative of two independent experiments. **(C)** T cell activation with SIY peptide loaded T2-K^b cells and T cells shown in (A). Cell lines as indicated. Levels of IL-2 release with OVA peptide loaded cells were below background (data not shown). Data is representative of two independent experiments.

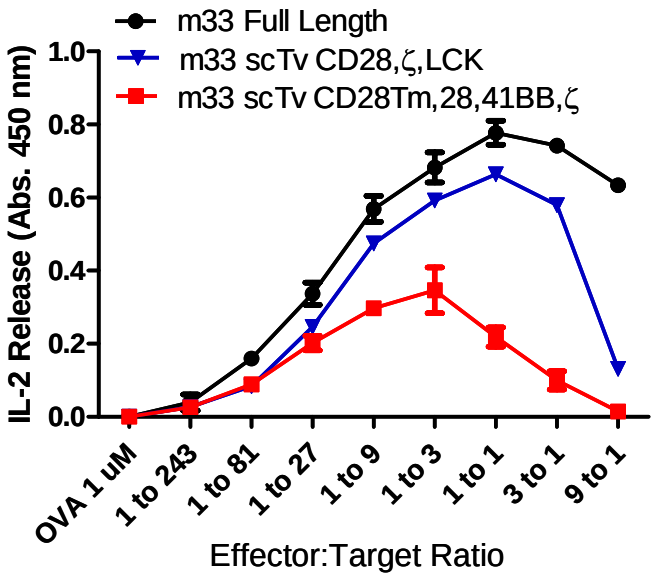

Figure 4.9. Activation of CD28Tm scTv CAR Constructs and Full-Length TCR with Excess SIY Peptide. m33 full-length TCR (black), m33 scTv CD28, CD3ζ, LCK (blue), and m33 scTv CD28Tm, CD28, 4-1BB,ζ (red) were incubated with target T2-K^b cells pulsed with 1 μM SIY peptide. A similar trend for T cell activation was observed with 100 nM SIY peptide, with T cell activation in the order of full length TCR>LCK scTv>4-1BB scTv, with lower levels of maximal IL-2 release for all cell lines (data not shown).

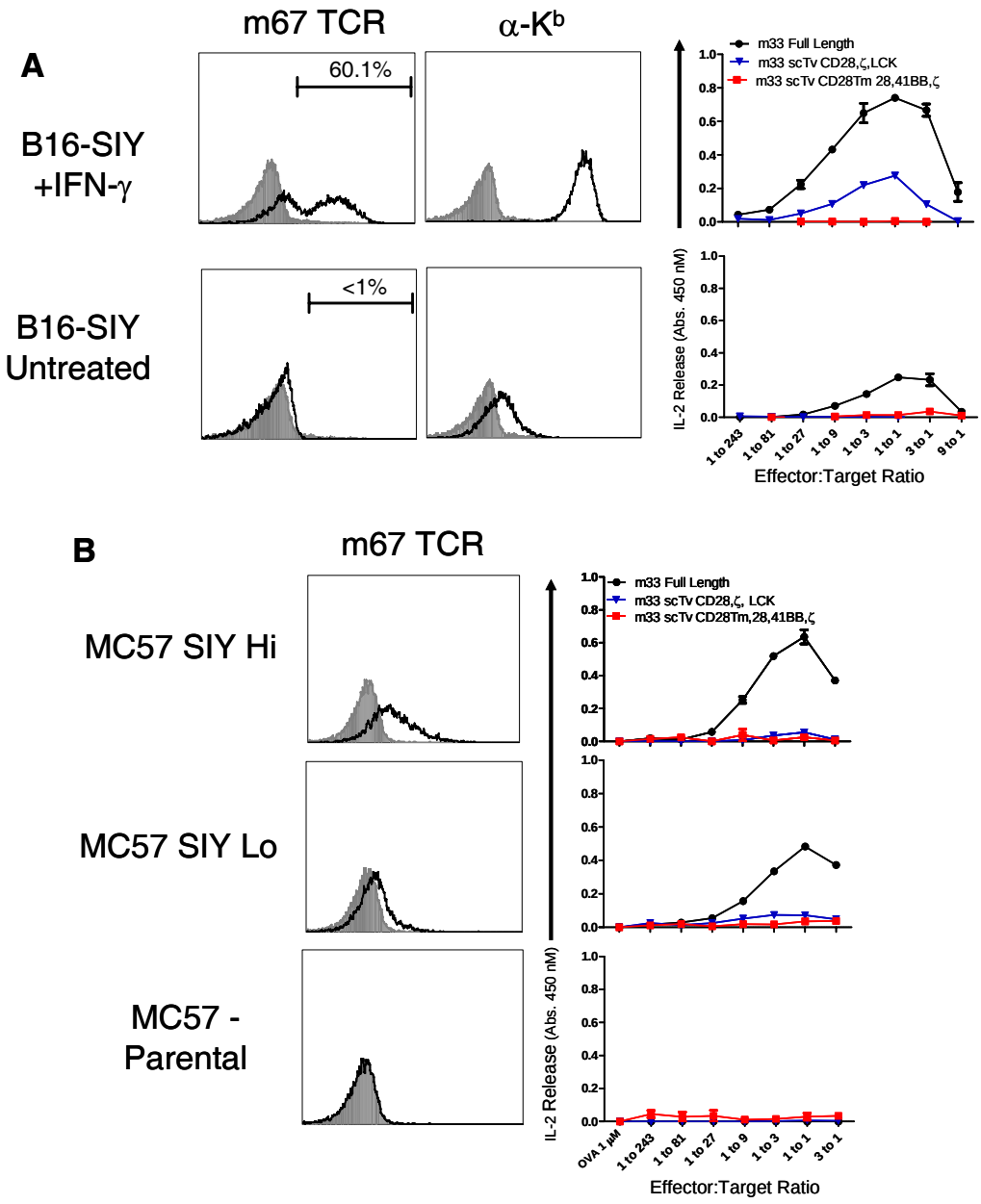

Figure 4.10. Activation of CD28Tm scTv Constructs and Full-Length TCR with Tumor Cells. (A) Activation of B16-SIY melanoma line (B16) with (top row) or without (bottom row) IFN-γ treatment 24 hrs before activation assay. Cells were analyzed by flow cytometery for SIY/K^b surface expression with m67 high affinity scTCR or anti-K^b. m33 full-length TCR (black), m33 scTv CD28, CD3ζ, LCK (blue), or m33 scTv CD28Tm, CD28, 4-1BB, CD3ζ (red) T cell activation as shown. **(B)** m67 high affinity TCR stain of MC57 fibrosarcoma cell lines expressing varying levels of SIY antigen. Activation data for corresponding cell line at right with colors corresponding to T cell lines as in (A).

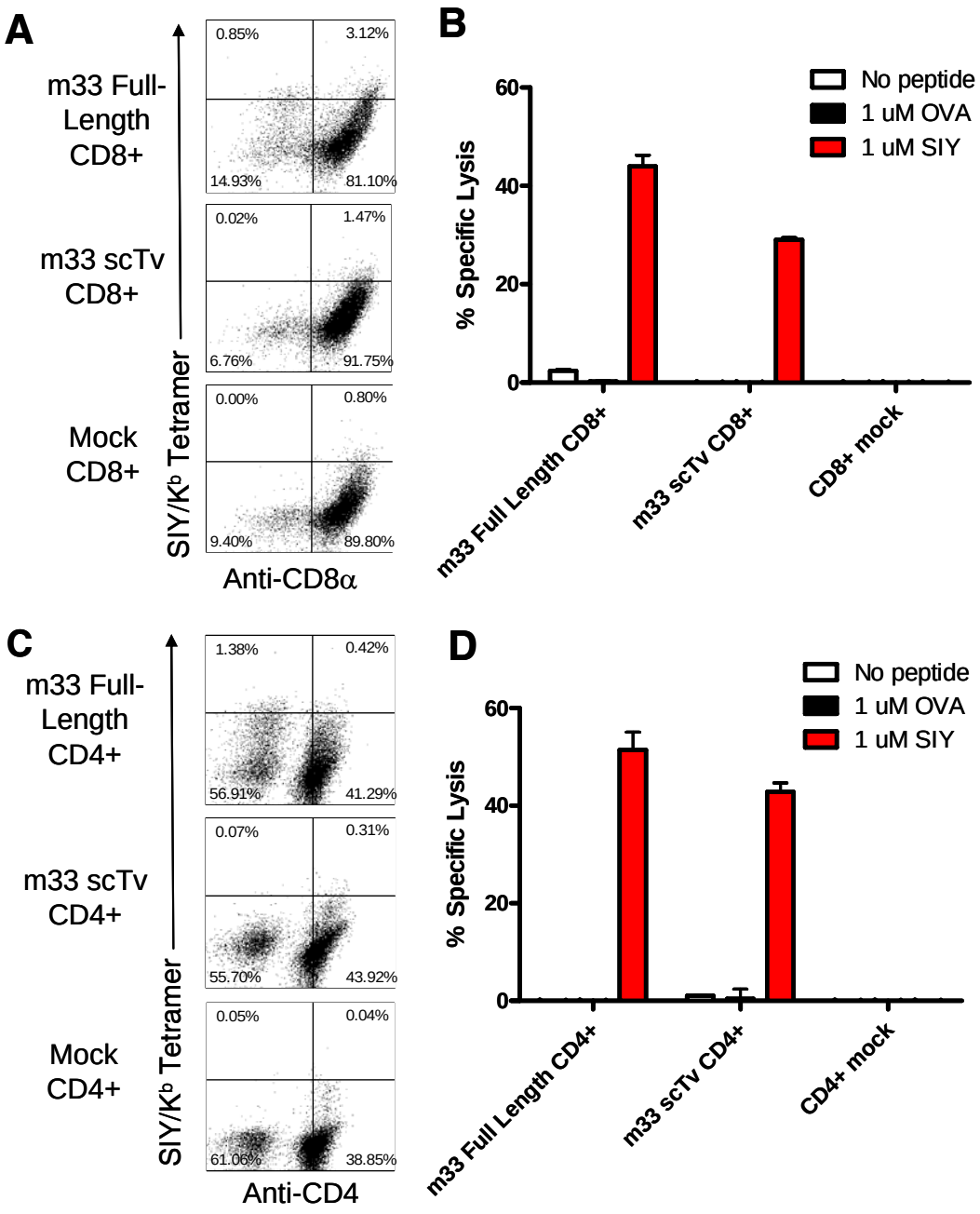

Figure 4.11. Transduction of Primary Murine T cells with m33 Full-Length or m33 scTv (CD28,4-1BB,CD3ζ) and Cytotoxic Activity of T cells. (A) Transduced CD8+ primary T cells were assessed for binding to SIY/K^b phycoerythrin tetramer (full-length TCR or scTv expressing cells, Y-axis), and CD8 surface levels (anti-CD8α, X-axis). Percentages of cells in each quadrant as indicated. **(B)** T cells killing of target cells pulsed with either 1 μM SIY peptide (red), 1 μM OVA peptide (black) or no peptide (white). **(C)** Transduced CD4+ primary T cells were analyzed as in (A). **(D)** T cell killing with CD4+ cells from (C). Experiments in B and D are representative of two independent experiments, performed by Dr. Adam Chervin.

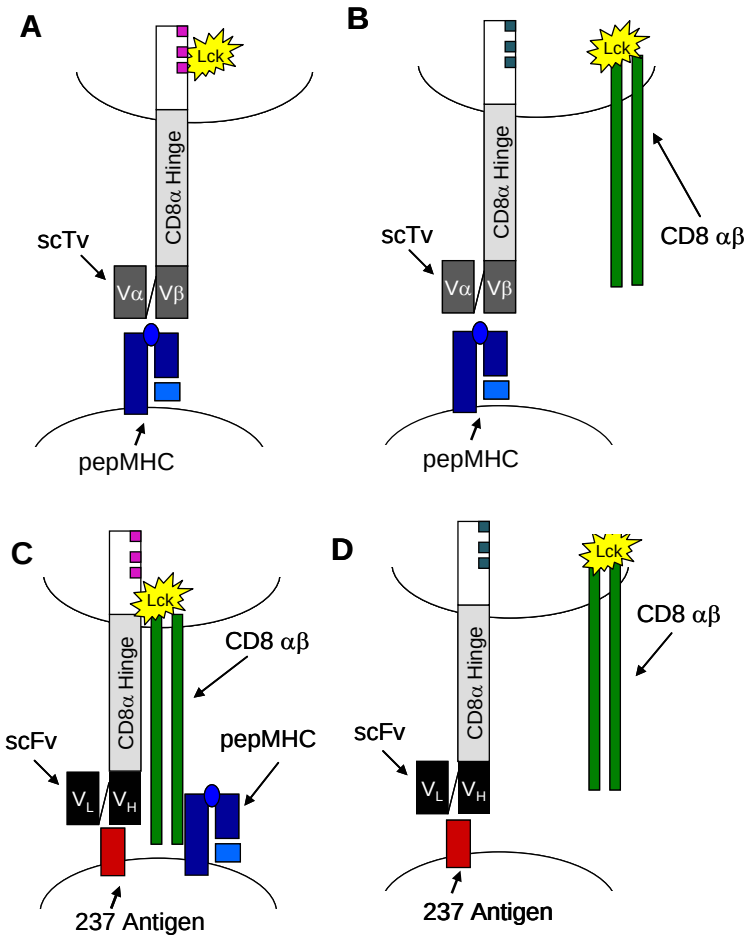

Figure 4.12. Model for scFv Cooperativity for Recognition of Non-MHC Restricted Antigens. (A) The scTv CAR (Dk. gray, antigen binding domain) recognizes SIY/K^b pepMHC (blue, as indicated). Intracellular LCK ('free' LCK, yellow) that is not fused to the CAR can associate with ITAMs (immune tyrosine activation motifs), and phosphorylation occurs at these ITAMS in CD3ζ (magenta) by 'free' LCK. **(B)** In the presence of CD8 (green), 'free' LCK associates with CD8. In the case of the m33 scTv, CD8 cannot readily associate with the same antigenic pepMHC as the scTv, and CD8 sequesters LCK away from the synapse. ITAMs are not efficiently phosphorylated (gray squares) because CD8 is inefficient in the recognition of neighboring MHC molecules, since the pepMHC is at a fixed orientation and distance from the membrane. **(C)** Recognition of 237 antigen (red) by the 237 scFv (black) can be enhanced by CD8 recognition of neighboring pepMHC molecules (blue) on tumor cells. CD8 is sequestered to the TCR:tumor cell interface permitting phosphorylation of ITAMs (magenta). (D) In tumor cells that downregulate MHC, 237 scFv can still bind to 237 antigens on the surface of T cells, but CD8 cannot associate because of loss of MHC surface expression. LCK is sequestered from the scFv:tumor cell interface, decreasing the potency of T cell activation.

CHAPTER FIVE

ENGINEERING MOUSE HIGH-AFFINITY T CELL RECEPTORS AGAINST WILM'S TUMOR ANTIGEN USING T CELL DISPLAY

Introduction

A promising approach to tumor elimination involves the use of adoptively transferred T cells directed against tumor associated peptide-MHC complexes. Regression of tumors using adoptive immunotherapy has been demonstrated successfully subsequent to lymphodepletion in melanoma patients (128-131). In these studies, the persistence of T cell populations specific for cancerous tissue was observed one month after transfer (128). The potential value of T cell adoptive therapies is that they provide a route to target cancer tissue specifically, while generating a T cell memory population that could eliminate residual disease. Antigens targeted by adoptively transferred T cells, a tumor associated peptide-MHC complex, can include overexpressed self peptides or mutated peptides. Because CTLs against "self" peptides are deleted in the thymus during T cell development, individuals may be left with a T cell repertoire that has a reduced capacity to eliminate the cancer tissue. The absence of these T cells represents a major problem with many T cell based strategies: that is, *an individual may lack appropriate anti-tumor T cells due to tolerance mechanisms that operate against self-antigens*. T cell expansion *ex vivo*, followed by *in vitro* T cell receptor (TCR) gene transfer, may overcome this problem by introducing TCRs that are specific for these overexpressed antigens.

Recently, it was demonstrated that gene transfer of a melanoma specific TCR, directed against the MART-1/HLA.A2 antigen, into peripheral blood leukocytes and subsequent adoptive transfer back into patients with advanced melanomas led to cancer regression in 2 of 15 patients (131). A follow up study demonstrated objective regression in as high as 30% of patients receiving gene modified T cells specific for MART-1 or gp100 (132). These studies demonstrated the feasibility of using a TCR reagent that recognizes a tumor associated antigen, even in patients that fail to have

adequate tumor reactive lymphocytes in their T cell repertoire. However, the modest but significant clinical effects of the introduced TCR may in part have been related to the observation that the affinities of the TCR for MART-1/HLA-A2 or gp100/HLA.A2 were not optimized. Although the selected clones were considered to be of high avidity, the binding parameters of these receptors have not been characterized. We and others have demonstrated that monomeric binding affinity does not alone determine T cell functional avidity in recognition of tumor cells (304). Although the affinity of the TCR against the cognate tumor antigen should be optimized, it is also important that the affinity and cross-reactivity for self-peptide antigens be examined in order to avoid overt autoimmunity against low levels of self-pepMHC in non-malignant tissue. For instance, preclinical studies have demonstrated overt autoimmunity from introduced T cells in murine models due to recognition of self-antigen on non-target tissues by cancer-antigen specific T cells (305). In fact, in the clinical trials targeting melanoma by TCR gene transfer, a positive indicator of regression was often the loss of pigment in skin and hair follicles (306). Another complication from TCR transduced T cells is the mispairing (Chapter 4) of exogenous TCR chains with the endogenous chains, which can lead to lehal graft versus host disease (135),(136).

Although T cell targeting of melanoma antigens has been the most studied system, numerous other pepMHC antigens are overexpressed on other tumor types and these also need to be evaluated for TCR gene therapy. For example, the Wilm's tumor antigen (WT-1) is overexpressed in a variety of tumor cell types and represents an attractive target for adoptive immunotherapy for malignancies aside from melanoma. The Wilm's tumor protein is a zinc-finger transcription factor that has been predicted to have an oncogenic role in developed cancerous tissue (Reviewed in (307)). Isolation and expansion of WT-1 specific T cells demonstrated that CTL killing of peptide WT-$1_{126\text{-}134}$/HLA.A2 positive tumor cells was dependent upon the presence of IFN-γ (308). This finding implies that a low extent of killing by T cells in the absence of IFN-γ was due to low pepMHC surface levels. As stated by Stauss and colleagues in their article regarding WT-1 specific TCR detection in early breast cancer in the context of adoptive therapies "*strategies that allow high level of*

peptide/MHC complex presentation and/or improved CTL avidity may be of clinical benefit (308)*"*. This makes the WT-1 tumor antigen a strong candidate for development of high affinity T cell receptors, as $CD8^+$ T cells with higher affinity receptors may be able to recognize fewer pepMHC complexes on the antigen presenting cell surface (206). In addition, recruitment of $CD4^+$ T cells through a high-affinity, class I restricted (WT-1/MHC) TCR could promote enhanced IFN-γ secretion as has been observed in adoptive transfer model systems (225). The ability of class-I specific high-affinity TCRs to mediate killing in $CD4^+$ cells has also been shown, further illustrating the value of a potent $CD4^+$ TCR response (309). Increased cytokine release in the tumor microenvironment is crucial for maintenance of $CD8^+$ T cells, as tumor infiltrating lymphocytes often lose cytotoxic function in the absence of $CD4^+$ T cell help (310).

An immunodominant peptide of WT-1 ($WT\text{-}1_{126\text{-}134}$, RMFPNAPYL) has been demonstrated to be expressed on the surface of tumor cells in the context of the human MHC human leukocyte antigen A2 (HLA.A2) in multiple tumor types (311),(312),(313), (314). WT-1 derived peptides presented by HLA.A2, the most common class I allele present in 40-50% of the population, have recently been shown to promote clearance of acute myeloid leukemias (AML) in clinical trials when the peptides were introduced by mRNA electroporation into dendritic cells in a vaccine format (315). This study demonstrated that in AML there was sufficient antigen on tumor cells to promote immune clearance given sufficient priming of WT-1 specific T cells and validates WT-1 as a target for immunotherapy. The murine homolog of the WT-1 peptide is identical to the human HLA.A2 binding peptide, and is presented by the murine MHC allele D^b. Consequently, murine tumors that overexpress WT-1 could provide a comparable model system to explore the efficacy of T cell treatments with high-affinity receptors and thus better define the optimal range of affinities for targeting overexpressed self-antigen. There is also a need for a model murine WT-1 system, as most model systems for WT-1 TCR gene transfer have used humanized mice which do not allow for the evaluation of potential autoimmune effects when targeting a self-antigen (316),(317).

The focus of the current study was to engineer high affinity T cell receptors against a murine peptide-MHC WT-1/D^b. To improve T cell responses to tumor antigen, we and others have engineered T cell receptors for enhanced affinity to defined antigens using yeast, phage, or T cell display (Reviewed in (146)). Yeast display and phage display are limited by the potential instability of full-length or single-chain TCRs, as evidenced by the fact that only a handful of TCRs have been engineered for enhanced affinity using phage or yeast display (157),(154), (148),(150),(149). Although rational design has been used to raise the affinity of TCRs 100-fold (318), to date these methods have required structural information about the lower affinity TCRs in complex with pepMHC, an approach that is also hindered by difficulties in expressing and crystallizing full-length TCRs in complex with antigens (reviewed in (59)).

Recently, we developed a T cell display platform using the $58^{-/-}$ T cell hybridoma to display TCR variants, and select for high-affinity T cell receptors using pepMHC multimers in combination with fluorescence activated cell sorting (147). The T cell display system allows for the generation of libraries on the order of 10^5 to 10^6 variants without the need for additional *in vitro* engineering to assure TCR is cell surface expressed. Here, I used a TCR called 3D that is specific for the model murine Wilm's tumor antigen (WT-1, peptide RMFPNAPYL) in the context of the MHC D^b, to isolate enhanced affinity TCR variants of the 3D TCR. The variants were capable of redirecting T cells independently of the coreceptor CD8. This chapter describes the refinement of T cell display to allow for isolation of high-affinity variants of the 3D TCR, as well as highlights the possibility of using murine high affinity mutations to direct the engineering of human TCRs against an analogous human WT-1 antigen.

Materials and Methods

Peptides, Antibodies, Dimer Reagents, and Cell Lines

Peptide gp100 (EGSRNQDWL) and WT-1 (RMFPNAPYL) that bind to the MHC allele D^b were synthesized by the Macromolecular Core Facility at Penn State

College of Medicine (Hershey, PA). Peptides were purified by reverse-phase chromatography using a C18 column with mass confirmed by MALDI.

The following antibodies and peptide:MHC multimers were used: PE-conjugated anti-mouse Vβ10 (Clone B21.5, eBiosciences), DimerX D^b (BD Biosciences), PE-conjugated anti-mouse CD8α (Clone 53-6.7, BD Pharmingen), Goat anti-Mouse IgG AlexaFluor 647 (Molecular Probes) or AlexaFluor 488, and PE-conjugated anti-Cβ (Clone H57-597). DimerX D^b was loaded with 120-fold excess peptide of interest at 37 degrees C for 16 hours as outlined by BD biosciences protocol.

PLAT-E (Clonetech) retroviral packaging cell line was maintained in DMEM with 10% FCS, L-glutamine, penicillin and streptomycin. T2-D^b, a TAP-deficient cell line that presents exogenous peptides, the EL4 tumor line and $58^{-/-}$ T cell hybridoma were maintained in RPMI 1640 complete medium supplemented with 10% FCS, L-glutamine, penicillin and streptomycin. The $58^{-/-}$ T cell line, with or without CD8α/CD8β, was transduced with retroviral supernatants of the full-length TCR mutant constructs described as previously described (153).

Creation of 3Dα-P2A-3Dβ TCR Construct

The 3D TCR insert was amplified from the codon-optimized (GeneArt) 3Dα-P2A-3Dβ (Vα3, Vβ10) construct in the pMig2R cloning vector (Greenberg Lab). An AgeI site and MluI site were introduced at the 5' and 3' end respectively by PCR, and the 3D P2A TCR insert was introduced into the MSCV retroviral vector which previously contained the 2C TCR cassette as has been described (Chapter 3).

Library Creation and FACS for Isolation of Improved Affinity 3D TCR variants

The 3D TCR in MSCV was used as a template for the creation of three separate degenerate amino acid libraries, each of three codons in length, spanning CDR3α (Sequence: TAPMDSNYQ). The mutated TCR insert was generated by overlap extension PCR, and the inserts with the three amino acid degeneracy were subsequently cloned into the MSCV vector using AgeI and BspEI restriction sites. The BspEI site is within the Cα of the 3D TCR. Ligation gave libraries of at least

7,000 transformants (Table 1), with at most 20% of library as unmodified, background 3D wild type TCR. The DH5α cell libraries were expanded and plasmid DNA was isolated. Library DNA was introduced into the PLAT-E packaging cell line using lipofectamine, and 24 hours after packaging cells were detoxified (removal of supernatant from transformation) and resuspended in 6 mLs RPMI + L-glutamine. 24 hrs later retroviral supernatants were harvested. Viral supernatants were spinfected on top of $1X10^6$ $58^{-/-}$ cells and cells were sorted as described (Results).

Identification of CDR Mutations that Improve Affinity for WT-1 from L3 Library

Total RNA was isolated (Qiagen RNeasy kit) from the L3 library after 3 sorts (3D L3). RNA was converted to cDNA (Biorad, iScript cDNA kit) and then subjected to two rounds of nested PCR. The PCR product was sequenced, yielding two species that were predicted to respresent separate products: a predominant sequence, called Q103H (CDR3α: TAPMDSNY**H**), and the minor sequence, called N101P, Q103Y (CDR3α: TAPMDS**P**Y**Y**).

Characterization of Q103H and N101P, Q103Y Cell Lines

The mutations from the two PCR products from the antigen-selected 3D L3 library were introduced into the 3D MSCV vector by Quikchange mutagenesis (Stratagene) using the exact nucleotides that were isolated from nested PCR. The two vectors were then transfected into the PLAT-E cell line, and the supernatant was used to transduce the $58^{-/-}$ T cell hybridoma line that lacks the CD8 coreceptor. Cell lines were characterized for binding to WT-1/D^b DimerX (BD Biosciences). The gp100/D^b DimerX was used as a control.

Results

T Cell Display of a Murine WT-1 Specific TCR

The 3D murine T cell receptor was introduced into the MSCV retroviral vector (schematic, Figure 5.1A), and subsequently used to tranduce the $58^{-/-}$ T cell hybridoma line. Cells were sorted to express >95% Cβ positive T cells. Notably, 3D

TCR^+ cells that lacked CD8 did not bind to WT-1/D^b dimer on ice at detectable levels (Figure 5.1B, top panel). To improve the affinity of the WT-1 specific TCR, I generated three different T cell display libraries in CDR3α (complementary determining region 3) as performed with the 2C T cell system (147). Previous engineering projects have demonstrated that high affinity binding solutions often exist within CDR3α (157), (101). Moreover, we and others have shown modest increases in affinity for pepMHC by single or dual amino acid changes in TCR CDR regions (319), (320),(321),

Hence, I generated several three-codon libraries, designated as L1, L2, and L3, that spanned the CDR3α loop of the 3D TCR (Figure 5.1A) using the T cell hybridoma $58^{-/-}$ to display the mutated TCRs. The ligated plasmids were transformed into *E. coli* and the number of independent transformants was calculated by plating a sample of the transformants, prior to preparation of the mutated pool of plasmids (Table 5.1). The purified plasmid preparations were transfected into PLAT-E cells for the production of packaged viruses. T cell hybridoma cells were transduced with the packaged viruses, and CDR3α libraries were analyzed for anti-Cβ cell surface expression after 4 days in culture (data not shown). From the per cent of cells that were positive, and the total number of $58^{-/-}$ cells, it was possible to calculate the total number of independent transductants for each library (Table 5.1). From these calculations, it was possible to deduce that the limiting factor in final $58^{-/-}$ library size (range from 7×10^3 to 3.6×10^4) was not T cell transduction but was transformation of the ligated, retroviral vector into *E. coli* (Table 5.1). Our lab has subsequently used *in vitro* enzymatic reaction to improve this transformation efficiency for other TCRs and can currently generate greater than 1×10^6 variants by homologous recombination, sufficient to cover the theoretical diversity of a three amino acid degeneracy library (data not shown).

After the 3D L1,L2, and L3 libraries were introduced into the $58^{-/-}$ line (Table 5.1, efficiency of transduction), cells were sorted one time with anti-Cβ antibody to obtain populations that expressed greater than 50% $C\beta^+$ T cells (Figure 5.1B, and S1 in Figure 5.2). The sorted cells were expanded in culture, and analyzed by flow cytometry for ability to bind to the WT-1/D^b dimer at 125 nM (Figure 5.1B). The 3D

L3 library (wild type: CDR3α: NYQ) appeared to contain a small population of T cells that bound to WT-1/D^b dimer at 125 nM (0.56% in APC positive gate for L3 compared to 0.19% average for 3D wild type TCR, L1, and L2). The L1, L2, and L3 libraries were sorted once with WT-1/D^b dimer at 300 nM (Sort 2); however, only a positive population which bound WT-1/D^b dimer was observed for the L3 library (Figure 5.2, S2 cells after 1 dimer sort). I chose to pursue this L3 library and sorted it a second time with WT-1/D^b dimer at 200 nM (Sort 3, L3 S3). After two sequential rounds of sorting with the WT-1/D^b dimer (S2 and S3, Figure 5.2), the L3 library contained a population that bound to WT-1/D^b dimer with a 13-fold increase in mean fluorescence intensity (S3 population), despite expressing equivalent surface levels of anti-mVβ10 (Figure 5.2). Thus, the L3 library yielded a T cell line with improved affinity for WT-1/D^b relative to the wild-type 3D TCR.

Isolation of CDR3α Sequences With Enhanced Affinity for WT-1/D^b

To determine the specific TCR mutations that improved affinity for WT-1/D^b, RNA was isolated from the L3 library T cell line after the second sort with dimer (L3 S3 in Figure 5.2) and cDNA was generated. Nested PCR using α-chain primers was performed from cDNA template. The DNA sequence of the PCR product revealed two predominant sequences (Figure 5.3) that differed from the 3D wild type at codons 101 and 103. Based on the intensity of the predominant species, the sequence yielded a single mutation of the 103 codon from CAG to CAC (a Q103H mutation). Based on the minor species in the sequence, there were two mutations, one at codon 101 from AAC to CCC (a N101P mutation) and the other at codon 103 from CAC to TAC (a Q103Y mutation). Because the relative intensities of the sequences for the minor species were about equal, I hypothesized that a single double mutant N101P/Q103Y may represent this clone. While it was possible that other solutions existed within the 3D L3 line that are even more minor products, the predominance of these two sequences prompted me to explore the single (Q103H, called NYH for the sequence of this region) and double mutant (N101P,Q103Y, called PYY for the sequence of this region) TCRs, and the effects of these mutations on affinity and T cell function.

Single and Double Mutations in CDR3α Enhance Affinity for WT-1/D^b and the Double Mutant Allows for CD8-Independent T Cell Activation

The single (NYH) and double (PYY) mutations isolated from 3D L3 were introduced using "quick change" mutagenesis into the 3D MSCV vector, and the mutants were transduced into the 58$^{-/-}$ T cell line. Cell surface expression was monitored with anti-mVβ10 antibody (Figure 5.4A). The NYH TCR was expressed at about 2-fold higher surface levels than the 3D wild type TCR or the PYY variant in CD8$^-$ cells. Titration of WT-1/D^b dimer on the three cell lines revealed that the NYH and PYY mutants bound to DimerX at concentrations as low as 10 nM, with the double mutant having a slightly higher relative affinity compared to the single mutant (Figure 5.4B). The wt 3D TCR required almost 100-times more dimer to bind at relatively low levels.

The 3D CD8$^-$ T cell lines were incubated with various concentrations of the WT-1 peptide in the presence of TAP-deficient T2-D^b antigen presenting cells (APCs) to determine the impact of improved TCR affinity for antigen on T cell activation (Figure 5.4C). T cells that expressed the PYY mutant were activated efficiently by WT-1 peptide, but not by null peptide gp100 peptide (data not shown). The NYH mutant and wild-type 3D expressing cells were only activated at very high peptide WT-1 concentrations in the absence of CD8, suggesting that the affinity of the NYH expressing cells may be below the threshold for CD8 independence. In contrast, it is likely that the affinity of the active mutant, PYY, is above the CD8 independence threshold, and that it has a K_D value less than about 1 μM (102).

The Single and Double Mutant Mediate Enhanced Peptide Dependent Activation in CD8$^+$ T Cells

The NYH and PYY mutant WT-1 specific TCRs were introduced into CD8αβ$^+$ 58$^{-/-}$ T cells to assess the ability of the high-affinity mutations to function in the presence of the CD8 coreceptor. Surface expression of the TCRs were similar, as judged by staining with the anti-mVβ10 antibody and CD8 coreceptor levels were equally high among the lines, as judged by staining with an anti-CD8α antibody (Figure 5.5A). The CD8$^+$ cell lines were incubated with various concentrations of the

WT-1 peptide and the D^{b+} APC line EL4, and IL-2 release was measured. T cells expressing the NYH and PYY mutants yielded about a 10-fold improvement in antigen sensitivity, suggesting that the affinity of the wild type TCR is not optimized for CD8-enhanced T cell activity, and that the mutant TCRs might provide some advantage in the detection and lysis of target cells that express lower levels of the endogenous WT-1 peptide.

Endogenous Peptides Improve T Cell Sensitivity to WT-1 Peptide In the Presence of CD8

The surface of APCs contains many other peptides distinct from WT-1 that T cells normally must survey to identify target cells. To better define the role of endogenous peptides in the recognition of WT-1 by the T cell lines, two D^b expressing T cell lines, T2-D^b and EL4, were compared for their ability to stimulate 3D wild type TCR and the PYY mutant TCR. The T2-D^b cell line presents exogenous peptides, predominantly, due to its defect in TAP, the transporter for endogenously processed peptides. In contrast, the EL4 cell line presents a plethora of endogenous peptides in addition to the exogenously added peptides. In the absence of CD8, the 3D wild type TCR is not active with T2-D^b or EL4 (Figure 5.6A), presumably because the receptor is not of sufficient affinity to mediate activity without the synergistic binding of CD8. T cells with the affinity-engineered TCR PYY were activated by WT-1 peptide at similar sensitivities with EL4 or T2-D^b APCs (Figure 5.6B), suggesting that the endogenous peptides did not influence T cell antigen sensitivity in the absence of CD8.

In distinct contrast, CD8 expression allowed both the 3D wild type (Figure 5.6C) and PYY (Figure 5.6D) TCRs to mediate improved antigen sensitivity with the EL4 APC line relative to T2-D^b. This finding is consistent with the hypothesis that the endogenous peptides present on EL4 APCs act as coagonists, improving recognition of antigen. While one could argue that higher surface levels of D^b on EL4 versus T2-D^b could have cause improved WT-1 recognition, then one would have expected the PYY $CD8^-$ cells to show improved antigen sensitivity with EL4 cells relative to T2-D^b cells (Figure 5.6B). The phenomenon of coagonist activity has

been demonstrated by multiple groups and is thought to facilitate the proliferation of low affinity T cells specific for antigen during the course of infection (322),(323),(324),(325). While it is possible that this activity is the result of binding of self pep-MHC by the 3D and PYY TCRs, the results in the absence of CD8 suggest this is not the case (i.e. there was not an enhancement of activity with EL4). Thus, the enhancement in activity in the presence of CD8 appears to be due to CD8:self pepMHC interactions, perhaps similar to that observed with the 237 scFv CAR in Chapter 4.

Homology Between Human and Murine WT-1 Receptors

To successfully target WT-1 tumor antigen in humans, it will be necessary to raise human T cells specific for WT-1 peptide in the context of HLA.A2. Phil Greenberg's lab (U. Washington) has now isolated a number of human $CD8^+$ T cell clones that are specific for WT-1/HLA.A2, including clones CE4, CE10, P20, P22, P1 and P18. In addition, they have isolated another murine WT-1 specific TCR called mWT-1. Because the WT-1 peptide presented by the human MHC HLA.A2 is identical to the nonomer peptide presented in mice by MHC alleles D^b or K^b (WT-1 sequence: RMFPNAPYL), I sought to compare the sequences of murine and human WT-1 restricted TCRs.

Alignment of the variable domains of six human and murine TCRs specific for WT-1 peptide revealed that there are conserved residues among the complementarity determining regions (CDRs) of some of the TCR sequences (Figure 5.7, red indicates identity between at least one murine and one human TCR). The presence of identical residues between the mouse and human TCRs could either be due to evolutionary conservation at these positions, or their shared antigen (peptide) specificity. For example, it has been demonstrated that germline-encoded CDR1 and CDR2 residues may have evolved to contact disparate MHC molecules (326),(99). To assess this, any position where there were identical residues between the mouse and human WT1-1 specific TCRs were examined among all Vα and Vβ from mouse and human. Those positions which showed greater than 50% identity between species (Figure 5.7, shown in red) were eliminated from the analysis, leaving a subset of

residues that were shared between the WT-1 TCRs. I hypothesize that these remaining residues, predominantly located within CDR3α and CDR3β, may serve as the focus for peptide:TCR contacts that confer specificity of the interaction. Thus, one might predict that these residues could serve as points for engineering higher affinity that would also translate to the human system.

Two of the human TCRs, P20 and P22, use the Vα2 gene segment shown to be amenable to engineering by yeast display in Chapter 2, and work is underway to engineer single-chain TCR versions of these human WT-1 specific TCRs. The observed sequence identity among the WT-1 specific TCRs should guide these engineering efforts. A recent study demonstrated that there was some preference for specific TCR Vβ (TRBV 12, Vβ8) gene usage in response to WT-1 in patients with myeloid malignancies (327), but this Vβ gene segment was not used by any of the clones isolated to date by the Greenberg lab. An additional two human T cell clones, P1 and P18, contained homologous CDR3α regions to the 3D TCR (Figure 5.7). At the time of writing this thesis, our collaborators (Greenberg Lab, U. Washington) are attempting to introduce the 3D improved affinity CDR3α mutations into these human TCRs to see if these mutations also improve affinity for human WT-1 pepMHC. Other experiments in the Greenberg lab are examining the 3D single and double mutant TCRs to see if these receptors escape central tolerance mechanisms. These experiments will provide insight into whether the mutant TCRs will react with self-tissue and cause autoimmunity, and provide an additional model system to define the optimal affinity of TCR for adoptive transfer.

Discussion

Tolerance mechanisms limit the repertoire of T cells that are specific for many human tumor associated antigens. This process can leave the immune system unequipped to eliminate cancer cells. To improve the immune response to overexpressed-self cancer antigens, I sought to engineer T cell receptors for enhanced affinity to a defined murine tumor antigen, WT-1/D^b, which serves as a model for human cancers that express the same WT-1 peptide antigen. Using the mouse T cell

hybridoma line $58^{-/-}$ for the expression of libraries of mutants in the TCR, two high affinity TCR variants were isolated that bound to WT-1/D^b dimer independent of the coreceptor CD8 as detected by flow cytometry. Thus, the T cell display system could be used to isolate higher affinity variants in T cell system other than the well-characterized 2C TCR (147). In addition, one of the TCR mutants was shown to mediate CD8-independent activity against the WT-1 on target cells, a hallmark of a higher affinity TCR:pepMHC interaction.

Although the size of libraries obtainable by T cell display is two orders of magnitude lower than that achieved by yeast display, this system eliminates the need to create stable templates on which to engineer high affinity TCRs. This is because the TCRs are expressed with associated CD3 subunits, in the context of the T cell membrane. An additional advantage of the T cell display system is that the isolated T cell lines expressing high affinity TCRs can be assessed for functional activity without the need for subcloning, as is required for yeast or phage display. Others have demonstrated that single and dual amino acid changes can improve the affinity of TCR for pepMHC (319),(320),(321). The single most significant disadvantage of these studies is that they rely on the construction and examination of individual mutations, without the higher throughput afforded by libraries of mutants combined with a selection system. Thus the T cell display system provides a high-throughput ($>10^4$ mutants) approach that rapidly assesses and selects for combinations of advantageous binding mutations . Given that higher affinity TCR mutants can be obtained with relatively few mutations in some instances, three-codon libraries were created for the 3D TCR. Although I was unable to cover the diversity of a three-codon library (32^3) for two of the libraries (L1 and L2), the L3 library contained 91,000 T cell transductants. Consistent with the larger size of this library, high affinity variants were isolated only from the L3 library. It is possible that other high affinity solutions would have been isolated from the L1 and/or L2 libraries, if their size had allowed the entire sequence space to have been included. Further refinement of the T cell display technology has allowed for generation of libraries of larger size (as high as 1×10^6) using an enzyme that catalyzes recombination *in vitro* (Clontech, InFusion Reaction Kit, unpublished data). This avoids the need to clone individual

sequences by ligation into the MSCV vector, a process that often yields reduced efficiencies of transformation. For future engineering projects using the T cell display system, it is likely that the T cell transduction efficiency will thus limit the library size (not the step involving transformation of *E. coli* with the retroviral vector).

The system used in the current study produced two high affinity variants which were subsequently characterized in CD8-negative and CD8-positive T cells. Characterization of T cells that expressed the double mutant, PYY, revealed activity in the presence of WT-1 peptide loaded cells, even in the absence of CD8. The single mutant of 3D, however, required CD8 to redirect T cells to WT-1 target cells. These data suggest that the double mutant should be able to redirect the helper activity of $CD4^+$ T cells to a WT-1/D^b-postive tumor. In the presence of CD8, the single and double mutant receptors provided a 10 to 100-fold increase in half-maximal sensitizing dose to WT-1 peptide loaded cells relative to the template TCR. This increase in sensitivity is analogous to that seen with high affinity variants of the 2C TCR directed against L^d (153) and K^b (101), where increased affinity led to similar increases in activity in the presence of CD8.

There is likely an affinity range where self-reactivity will lead to deletion of T cells with high-affinity TCRs, as has been observed for high-affinity variants of the 2C TCR (Boris Engels, Schreiber Lab, U. of Chicago). Peripheral tolerance mechanisms prevent self-reactive T cells from persisting. The two high affinity variants of 3D provide an additional model system to explore the optimal affinity window for tumor antigen specific TCRs in adoptive therapy formats, where the TCRs can improve or redirect the activities of $CD8^+$ or $CD4^+$ cells and avoid tolerance. Experiments are underway in our collaborator, Professor Greenberg's lab, to determine: 1) the activity of the variants in primary T cells and 2) if the two high affinity variants escape central and/or peripheral tolerance. In development, T cells expressing self-reactive TCR (i.e. TCR with too high of affinity for self antigen) are deleted in a process called negative selection (central tolerance). The affinity threshold for negative selection has been suggested to be between 1 and 5 μM K_D (328). Others have demonstrated that the levels of CD8 vary during selection, and

that the level is tuned to contribute to binding to self-antigens and deletion of many would be tumor specific TCRs (329),(330).

A potential problem with an adoptive transfer regimen, where $CD4^+$ T cells are "redirected" with a high-affinity class I-restricted TCR, is the potential for the generation of $CD4^+$ regulatory T cells which could suppress the immune response to the antigen-positive tumor. A recent study has demonstrated that the affinity of the TCR can dictate the formation of regulatory T cells (331), and thus TCRs with a range of affinities need to be examined for their propensity to develop into Tregs in adoptive transfer models. The two high-affinity TCRs against self-antigen WT-1 described here, along with the wild type 3D TCR, should allow further examination of these issues, and will better define the optimal affinity TCRs for transfer into $CD4^+$ and $CD8^+$ T cells.

Isolation of 8 T cell clones raised against murine (2 clones) and human (6 clones) WT-1 tumor antigen led to the identification of a number of conserved residues within the CDR3 regions. Others have observed similar conservation in response to a specific viral or tumor antigen (reviewed in (332)), but there are no instances I am aware of where homology in the CDR3 loops existed for T cell receptors across species directed against the same peptide. The sequence similarity observed for these clones may provide insight into future affinity engineering of other WT-1 specific TCRs and the immune system response to this particular self-antigen.

Figures

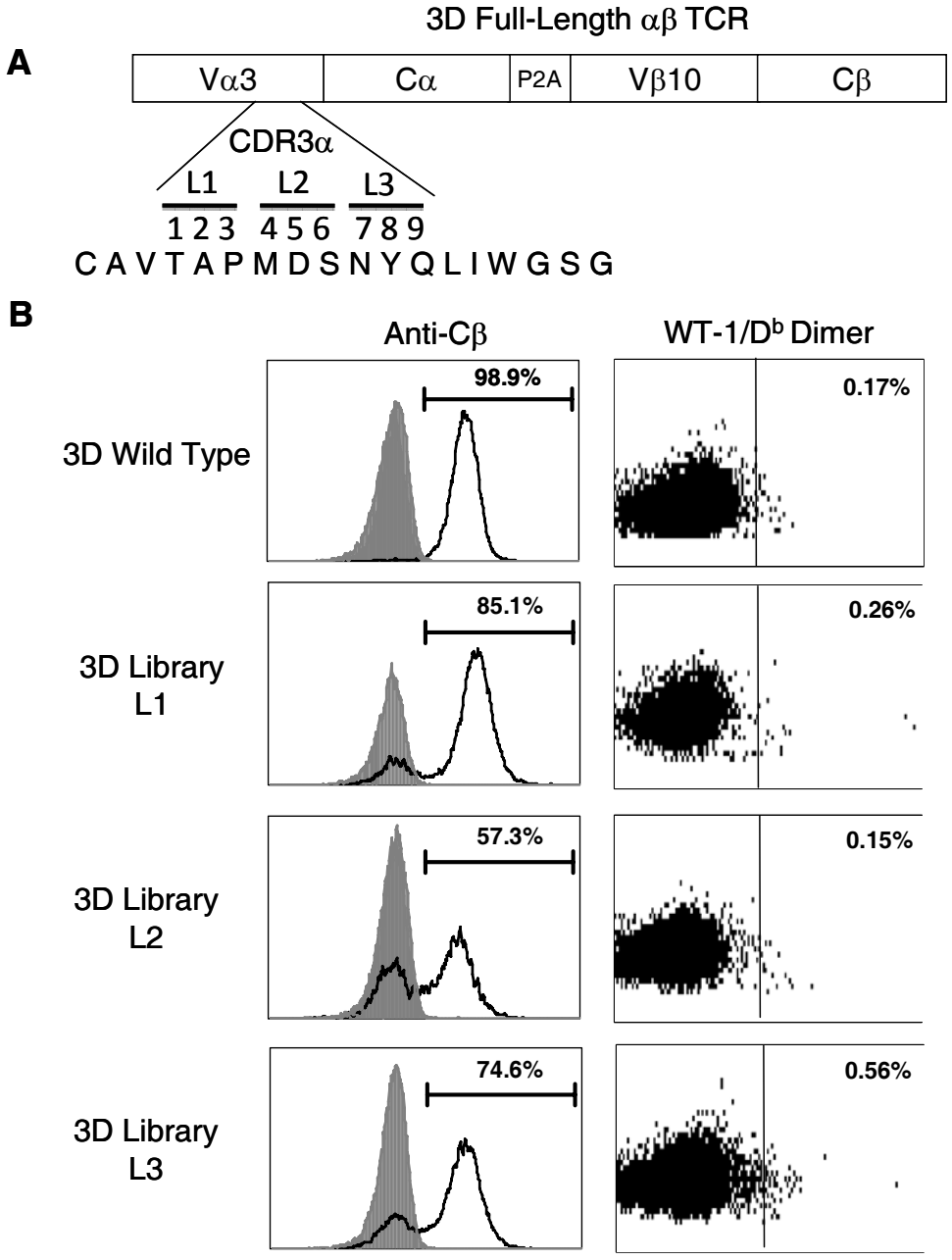

Figure 5.1. 3D Construct, CDR3α Library Design and T Cell Surface Expression. (A) Schematic of the 3D TCR construct. The CDR3α Region consists of 9 residues as highlighted. The nine residues were separated into 3 libraries respectively called L1, L2, and L3. **(B)** Surface expression of template TCR and CDR3α libraries L1, L2, and L3 as monitored by anti-Cβ after 1 round of selection with anti-Cβ. Notably, a population of DimerX WT-1 positive cells was present in the L3 library.

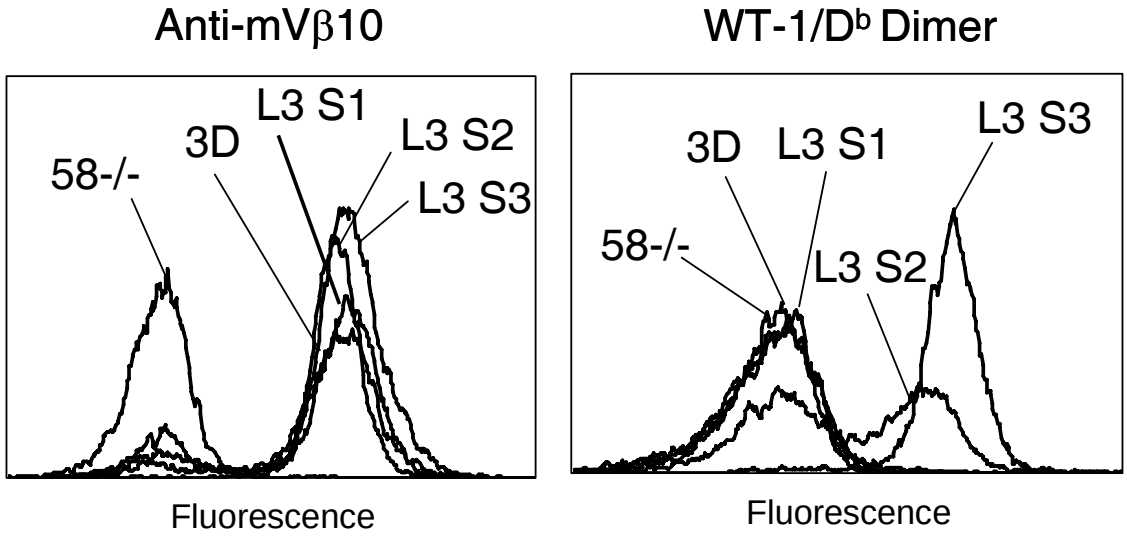

Figure 5.2. CDR3α L3 Library Surface Expression and DimerX WT-1/D^b Binding After Rounds of Selection. Cells were assessed for mVβ10 surface levels and WT-1/D^b binding after each sort as indicated. The 3D L3 Library (CDR789) was sorted with anti-Cβ for sort 1 (S1), DimerX WT-1/D^b at 200 nM for sort 2 (S2), and DimerX WT-1/D^b at 200 nM for sort 3 (S3).

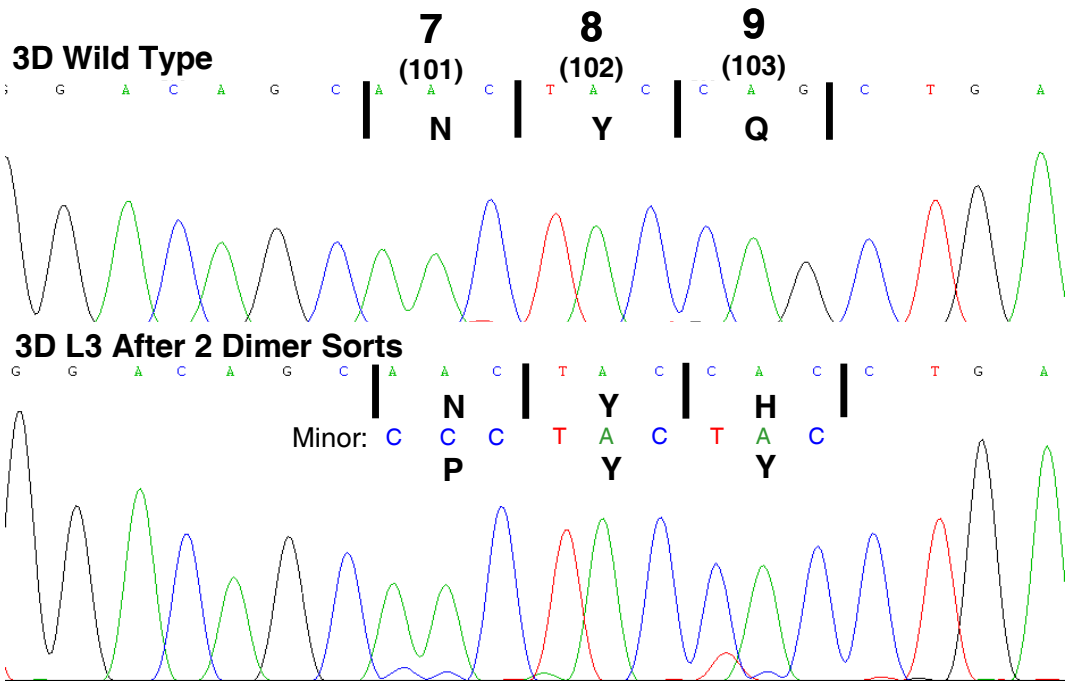

Figure 5.3. CDR3α DNA Sequence Analysis. DNA sequencing of the 3D full-length TCR template (NYQ) **(A)** and the L3 library after two sorts **(B)**. The L3 library contained a major species (NYH, single mutant) and minor species (PYY, double mutant) within the DNA sequencing trace.

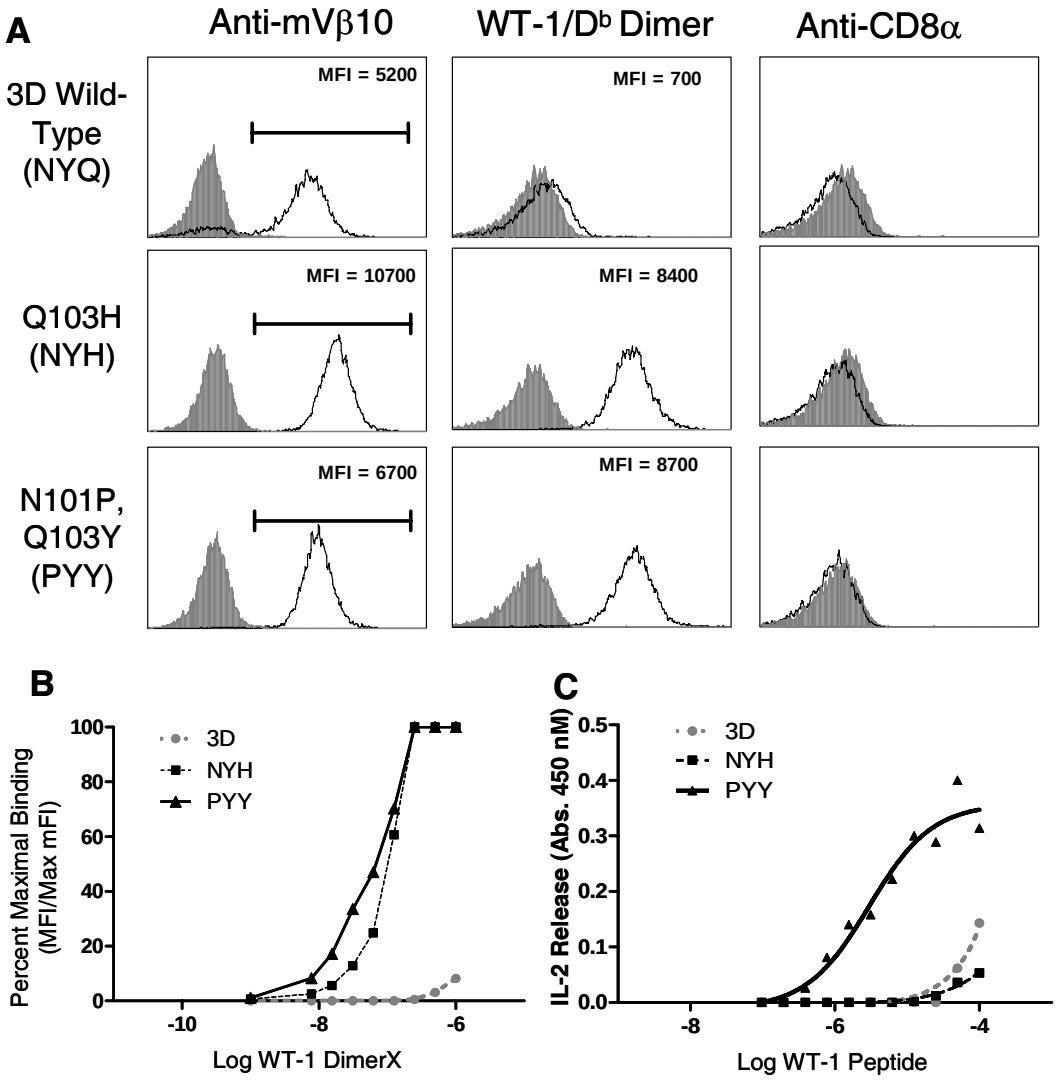

Figure 5.4. Characterization of Single and Double Mutant 3D TCRs in 58-/- T Cells. (A) Surface expression of 3D wild type (NYQ), single mutant (NYH), and double mutant (PYY). Staining with antibody or WT-1/D^b DimerX at 125 nM or antibodies is as indicated (black line) with 58-/- parental line as a control (gray histograms). MFI for Vβ is of the gate shown. For WT-1/D^b DimerX, MFI is expressed for the entire population of cells. **(B)** WT-1/D^b DimerX titration analysis by flow cytometry at 4 degrees C. **(C)** T cell activation in the presence of WT-1 peptide titrated on T2-D^b cells with wild type, NYH, and PYY mutants of 3D.

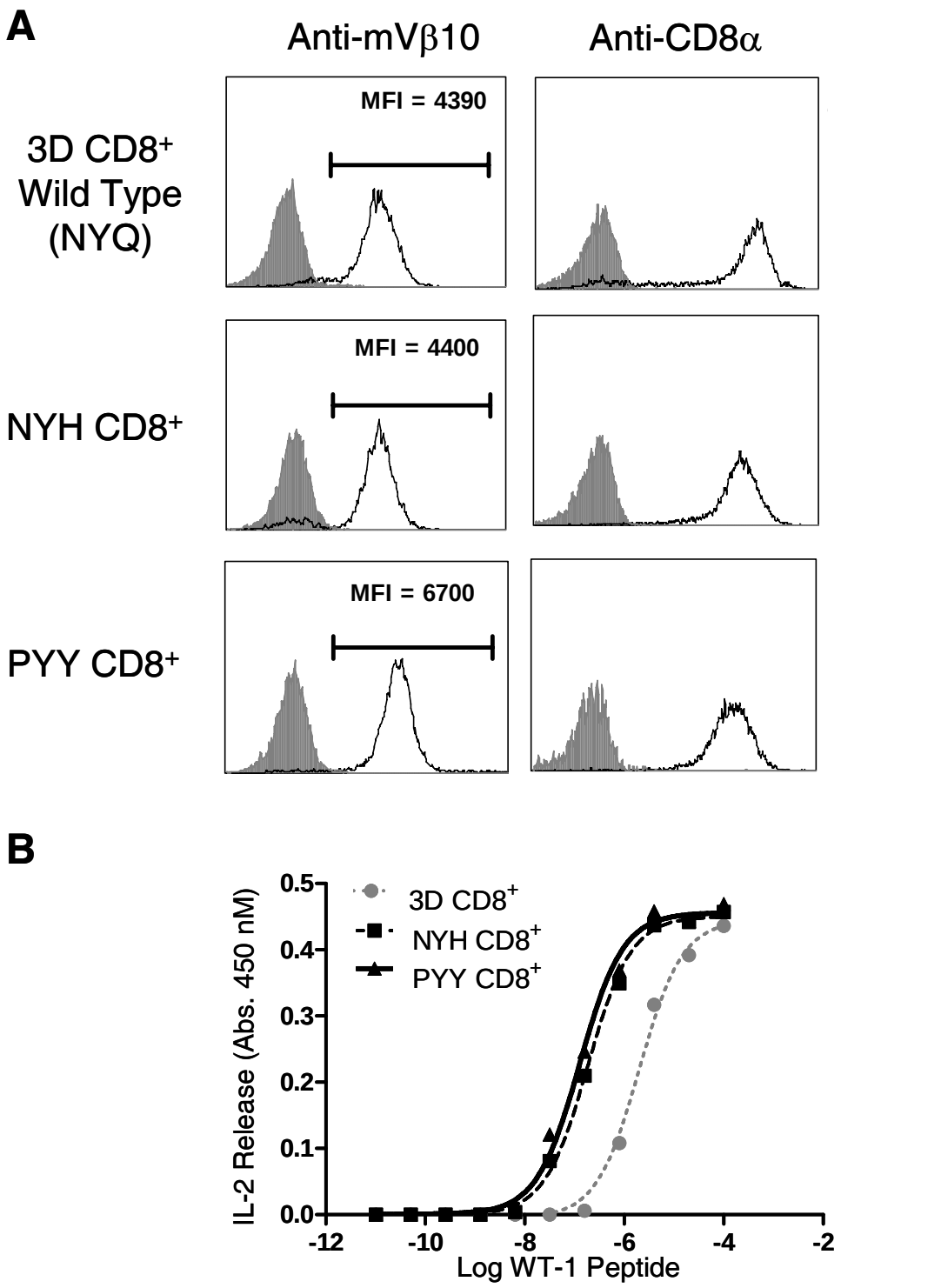

Figure 5.5. Characterization of NYH and PYY Mutants in CD8αβ^{+} 58$^{-/-}$ T cells. **(A)** Surface expression of NYH and PYY mutants in CD8^{+} cells as monitored by indicated antibodies. MFI for Vβ is for cells within the marker shown. MFI for WT-1/D^{b} DimerX is expressed for the entire population. **(B)** T cell activation of cells lines from (A) in the presence of WT-1 peptide loaded T2-D^{b} cells.

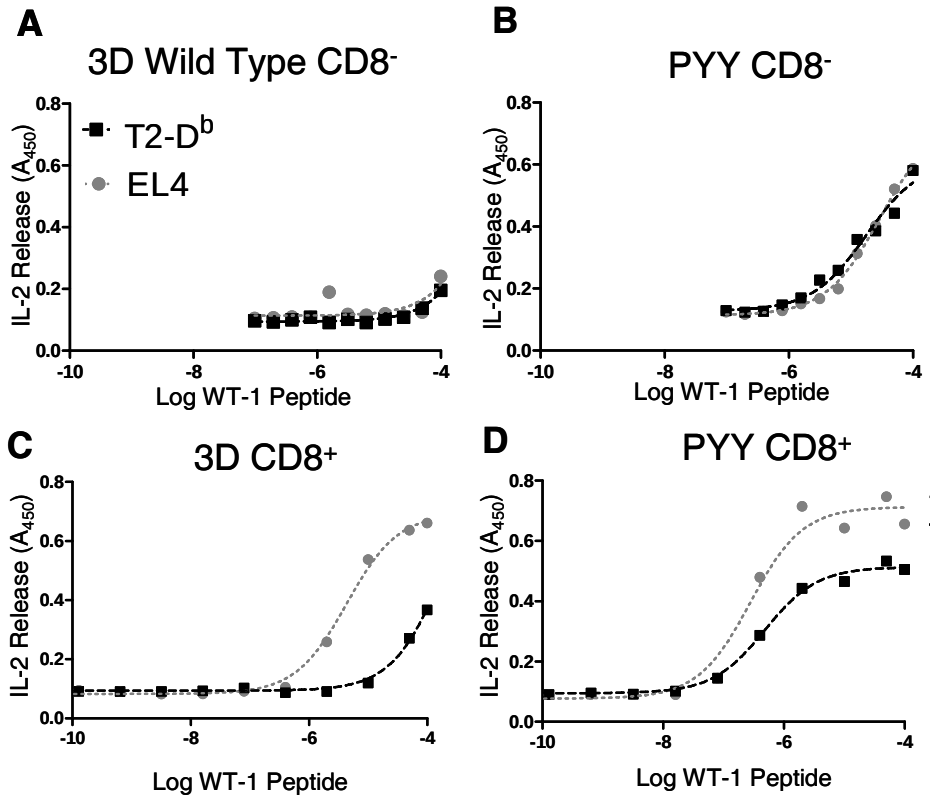

Figure 5.6. Comparison of Antigen Presenting Cell Lines with 3D and PYY Mutant. (A) $CD8^-$ T cells expressing 3D TCR or PYY mutant were incubated with either EL4 (gray) or T2-D^b (black) WT-1 peptide loaded cells. The EL4 cell line also presents other peptides, whereas the TAP-deficient T2-D^b cell line only presents exogenous WT-1 peptide. **(B)** The 3D TCR and double mutant (PYY) were expressed in $CD8^+$ T cells and incubated with either EL4 or T2-D^b as in (A). The coagonist activity of EL4 is apparent, as the sensitivity to WT-1 peptide was improved with EL4 relative to T2-D^b for both TCRs.

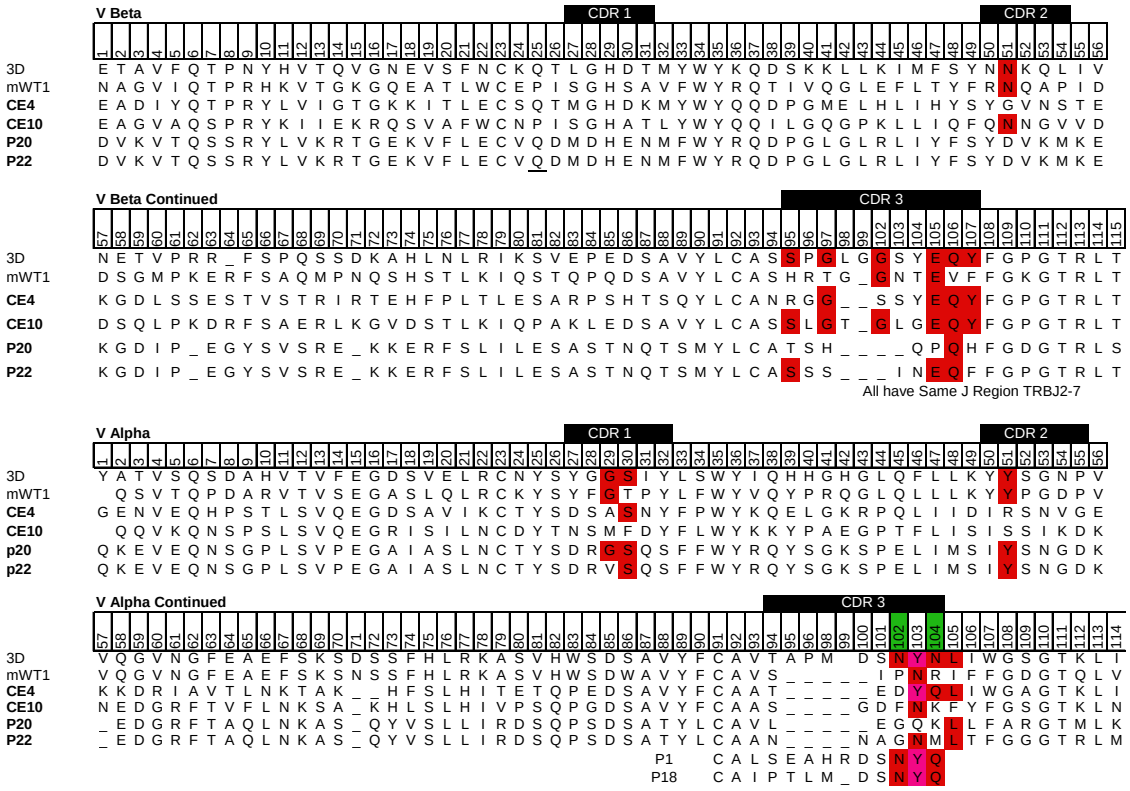

Figure 5.7. Alignment of WT-1 Specific Murine and Human TCRs. Alignment of murine (3D and mWT-1) and human (CE4, CE10, P1, P18, P20, P22) WT-1 specific TCRs. Red or pink: homology between at least one human and murine TCR. Green: Position of mutations that enhance affinity of the 3D murine TCR for WT-1/D^{b}. Residues that are conserved in more than 50% of mouse or human TCRs were eliminated from the analysis.

Table

Table 1. 3D CDR3α Library Sizes

Library	Transformants (*E. coli*)	T cell Transduction Efficiency (Percent, # of cells Transduced)	Library Diversity
L1 (TAP)	25,900	13.3% (133,000)	25,900
L2 (MDS)	7,000	2.5% (25,000)	7,000
L3 (NYQ)	36,600	9.1% (91,000)	36,600

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antibodies specific for a large melanoma surface antigen. *Cancer Immunol Immunother* 59:1197-1209.
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CURRICULUM VITAE

David H. Aggen

EDUCATION

University of Illinois at Urbana-Champaign, Urbana, IL ***September 2010***
Ph.D., Department of Biochemistry, UIUC
Dissertation Title: Engineering Human Single-Chain T Cell Receptors

Illinois Wesleyan University, Bloomington, IL ***May 2005***
B.A., Department of Chemistry

RESEARCH EXPERIENCE

University of Illinois at Urbana-Champaign ***March 2006 to 2010***
Medical Scholars Program (MD/PhD)
Graduate Research Assistant, Dept. of Biochemistry, Urbana, IL
Advisor: Professor David M. Kranz, Philip A. Sharp Professor of Biochemistry

- *Engineering of Soluble T Cell Receptors for Targeting Applications*:
  - Identified a human gene family, the Vα2+ T cell receptor, that is amenable to engineering
  - Using yeast display, isolated stabilized scTv specific for HIV and HTLV antigens
  - Expressed soluble forms of these scTv receptors in *E. coli* for potential diagnostic and therapeutic applications
- *Development of T Cell Receptor Based Chimeric Antigen Receptors for Adoptive T Cell Therapy Models*
  - Generated chimeric antigen receptors with extracellular domains derived from TCR variable domains
  - Demonstrated that stable, high affinity, scTv molecules fused to intracellular signaling proteins could redirect T cells to specific antigens
  - Developed CAR receptors with extracellular domains derived from an antibody scFv for comparison with scFv fusions in murine tumor model systems.

Mayo Clinic College of Medicine ***June 2004 to August 2004***
Summer Undergraduate Research Fellow, Rochester, MN.
Department of Nephrology, Department of Biochemistry
Advisor: Dr. Rajiv Kumar, M.D.

- *Isolation and Characterization of Proteins Associated with Phosphate Wasting in vitro and in vivo*
  - Studied effects of phosphatonins, proteins associated with renal phosphate wasting in patients with normal levels of vitamin D, parathyroid hormone, and serum calcium
  - Quantified effects of phosphatonins on phosphate clearance in a rat model system

Illinois Wesleyan University ***August 2001 to May 2005***
Undergraduate Research Assistant, Bloomington, IL
Department of Biology, Department of Chemistry

- *Laboratory for Bacteriochlorophyll Research* *August 2004 to May 2005*
  Advisor: Prof. David Bollivar
  - Isolation and characterization of pigments involved in the bacteriochlorophyll protoporphyrin synthetic pathway

RESEARCH EXPERIENCE CONTINUED

- *Laboratory for Organic Synthesis Research* *May 2003 to April 2004*
  Advisor: Prof. Ram Mohan
  - Developed applications of bismuth compounds in organic synthesis (environmentally friendly organic synthesis)

- *Inorganic Chemistry Research Assistant* *August 2001 to April 2003*
  Advisor: Prof. Rebecca Roesner
  - Developed synthetic reactions using hexamolybdate ions

TEACHING EXPERIENCE

University of Illinois at Urbana-Champaign ***Spring 2006 to Present***

Graduate Teaching Assistant, College of Liberal Arts and Science, Molecular and Cellular Biology
College of Medicine - UIUC

Served as occasional lecturer, grader, and led discussion sections

- Molecular and Cellular Biology: Immunology (MCB408)
- College of Medicine: Medical Microbiology and Immunology

PUBLICATIONS

[1] **Aggen, D. H.**; Arnold, J. N.; Hayes, P. D.; Smoter, N. M.; Mohan, R. S. *Bismuth Compounds in Organic Synthesis. Bismuth Nitrate Catalyzed Chemoselective Synthesis of Acylals from Aromatic Aldehydes*. Tetrahedron. **2004**, *60*, 3675-3679

[2] Chervin, A.S.; **Aggen, D.H**.; Raseman, J.M.; Kranz, D.M. *Engineering Higher Affinity T cell Receptors Using a T cell Display System*. Journal of Immunological Methods, **2008**, 339(2), 175-184.

[3] Richman, S.A.; **Aggen, D.H.**; Dossett, M.L.; Donermeyer, D.L.; Allen, P.M.; Greenberg, P.D.; Kranz, D.M. *Structural Features of T cell Receptor Variable Regions that Enhance Domain Stability and Enable Expression as Single-Chain VαVβ Fragments*. Molecular Immunology, **2009**, 46(5), 902-916

[4] **Aggen, D.H.**; Chervin, A.S.; Insaidoo, F.K.; Piepenbrink, K.H.; Baker, B.M.; Kranz, D.M. *Identification and Engineering of Human Variable Regions That Allow Expression of Stable Single-Chain T Cell Receptors.* Protein Engineering Design and Selection, Accepted Oct. 4 2010.

Manuscripts in Preparation:
[5] **Aggen, D.H.**; Chervin, A.S.; Stone, J.D.; Engels, B.; Piepenbrink, K.H.; Baker, B.M.; Schmitt, T.M.; Greenberg, P.D.; Schreiber, H.; and Kranz, D.M. *Transduction of Single-Chain T Cell Receptors Lacking Constant Regions circumvents Mispairing with Endogenous TCR.*

[6] Stone, J.D.; **Aggen, D.H.**; Chervin, A.S.; Narayanan, S.; Schmitt, T.M.; Greenberg, P.D.; Kranz, D.M. *Specific Endogenous Peptides Act as Antagonists in the Absence of CD8 and Co-Agonists in the Presence of CD8.*

[7] **Aggen, D.H.**; Stone, J.D.; Engels, B.; Schreiber, H.S.; Kranz, D.M. *Contribution of CD8 to T Cell Activation with Chimeric Antigen Receptors Directed Against Non-MHC Antigens.*

[8] **Aggen, D.H.**; Schmitt, T.M.; Greenberg, P.D.; Kranz, D.M. *Engineering of High Affinity T Cell Receptors Specific for Wilm's Tumor Antigen Using a T Cell Display System*.

POSTERS AND PRESENTATIONS

Posters

[1] **Aggen, D.H.**, Richman, S.A., Raseman, J.M., Greenberg, P.D., Kranz, D.M. *In vitro Engineered T Cell Receptors for Potential Cancer Immunotherapy,*
Cell and Molecular Biology Training Grant 20th Research Symposium, November 2007, Urbana, IL.

[2] **Aggen, D.H.**, Richman, S.A., Raseman, J.M., Greenberg, P.D., Kranz, D.M. *In vitro Engineered T Cell Receptors for Potential Cancer Immunotherapy,*
University of Illinois Urbana-Champaign College of Medicine Research Symposium, April 2008, Urbana, IL.

[3] **Aggen, D.H.**, Richman, S.A., Raseman, J.M., Greenberg, P.D., Kranz, D.M. *In vitro Engineered T Cell Receptors for Potential Cancer Immunotherapy,*
Medical Scholars Program Annual Retreat, August 2008, Urbana, IL.

[4] **Aggen, D.H.**, Chervin, A.S., Raseman, J.M., Kranz, D.M. *Engineering High Affinity T Cell Receptors Using a T Cell Display System,*
Cell and Molecular Biology Training Grant Research Symposium, October 2008, Urbana, IL.

[5] **Aggen, D.H.**, Chervin, A.S., Bowerman, N.A., Baker, B.M., Greenberg, P.D., Kranz, D.M. *Engineering T Cell Receptors for Targeted Immunotherapy Applications,*
University of Illinois Urbana-Champaign College of Medicine Research Symposium, "Emerging Environment of Medicine Practice, Progress, and Policy," April 2009, Champaign, IL.

[6] **Aggen, D.H.**, Chervin, A.S., Richman, S.A., Raseman, J.M., Greenberg, P.D., Kranz, D.M. *Engineering T Cell Receptors for Targeting Immunotherapy Applications,*
29th Annual Medical Scholars Program Annual Retreat, August 2009, Urbana, IL.

[7] **Aggen, D.H.**, Chervin, A.S., Richman, S.A., Raseman, J.M., Greenberg, P.D., Kranz, D.M. *Engineering T Cell Receptors for Targeting Immunotherapy Applications,*
Biochemistry Department Annual Research Symposium, September 2009, Urbana, IL.

[8] **Aggen, D.H.**, Chervin, A.S., Bowerman, N.A., Baker, B.M., Greenberg, P.D., Kranz, D.M. *Engineering T Cell Receptors for Targeted Immunotherapy Applications,*
Cell and Molecular Biology Training Grant 22nd Research Symposium, October 2009, Urbana, IL.

[9] **Aggen, D.H.**, Chervin, A.S., Stone, J.D., Engels, B., Piepenbrink, K., Baker, B.M., Kranz, D.M. *Engineering High Affinity Single-Chain T Cell Receptors for Adoptive Therapy Applications,*
Keystone Symposium, "Molecular and Cellular Biology of Immune Escape in Cancer," February 7th-12th, 2010, Keystone, CO.

[10] **Aggen, D.H.**, Chervin, A.S., Stone, J.D., Engels, B., Piepenbrink, K., Baker, B.M., Schreiber, H., Kranz, D.M. *Engineering High-Affinity, Single-Chain T Cell Receptors for Cancer Adoptive Therapy Applications,*
University of Illinois Urbana-Champaign College of Medicine Research Symposium, "Emerging Technologies in Medicine: Innovation, Intervention, Implication," April 2010, Champaign, IL.

Presentations

[1] *Engineering Human Tumor Specific TCRs for Improved Stability and Affinity*. Cell and Molecular Biology Training Grant Seminar Series, UIUC, November 6th, 2007.

[2] *Engineering Human Tumor Specific T Cell Receptors.* Department of Biochemistry Graduate Student Seminar Series, UIUC, April 23rd, 2008.

[3] *Engineering Single-Chain T Cell Receptors for Cancer and Viral Immunotherapy.* Department of Biochemistry Graduate Student Seminar Series, UIUC, March 4th, 2009.

[4] *Engineering Single-Chain T Cell Receptors for Cancer and Viral Immunotherapy.* Cell and Molecular Biology Training Grant Seminar Series, UIUC, April 7th, 2010.

[5] *Engineering Single-Chain T Cell Receptors for Cancer and Viral Immunotherapy.* Department of Biochemistry Graduate Student Seminar Series, UIUC, February 3rd, 2010.

AWARDS AND HONORS

- Cell and Molecular Biology Training Grant Trainee (UIUC Institutional Award) (August 2007-August 2009)
- Best Basic Science Poster: College of Medicine Research Symposium (April 2009)
- Poster Award: Cell and Molecular Biology Training Grant Research Symposium (November 2007)
- Illinois Wesleyan Alumni Scholarship (August 2001-May 2005)
- Sigma Xi Scientific Research Honor Society (ISU/IWU Chapter, Spring 2004)
- Chemistry Council of Illinois Scholarship Recipient (Spring 2004)
- Illinois Wesleyan Varsity Athletics: Men's Cross-Country Letter Winner (Fall 2002)