Generation and Expression of High Affinity, Tumor Antigen-Specific Mouse and Human T Cell Receptors to Genetically Modify $CD8^+$ T Cells for Adoptive Immunotherapy of Cancer

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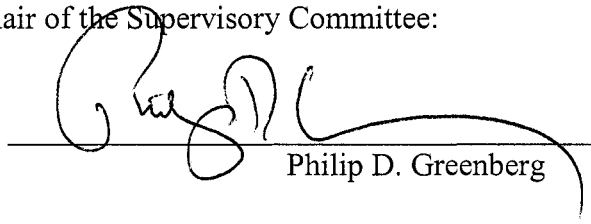

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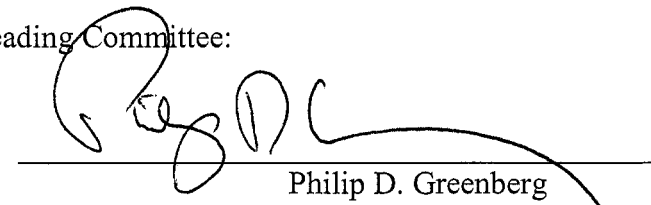

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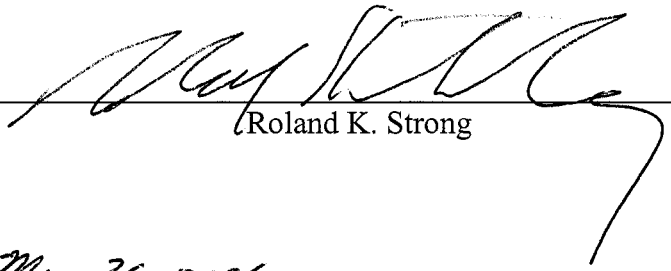

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Abstract

Generation and Expression of High Affinity, Tumor Antigen-Specific Mouse and Human T Cell Receptors to Genetically Modify $CD8^+$ T Cells for Adoptive Immunotherapy of Cancer

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Adoptive T cell immunotherapy has shown promise in treating some cancers, but the challenge of isolating T cells with high avidity for tumor antigens limits large-scale applicability. T cell receptor (TCR) gene transfer employing high affinity TCRs recognizing defined tumor-associated antigens can potentially circumvent these challenges. Using a well-characterized murine model of adoptive T cell immunotherapy for established malignancy, we have demonstrated the feasibility of eliminating disseminated leukemia using T cells genetically modified by TCR gene transfer.

We next examined the potential of targeting a clinically relevant tumor antigen, the transcription factor WT1, for which the expression patterns in normal and malignant cells are similar in mouse and man. WT1 is overexpressed in most leukemias and many solid tumors and its expression contributes to the malignant phenotype. The RMFPNAPYL peptide from WT1 is presented by HLA-A2 in humans, as well as by $H\text{-}2D^b$ in C57BL/6 mice, and has been shown to be the target of WT1-specific responses in both species. Like many tumor-associated antigens,

generating robust immune responses to the WT1 protein has been difficult as endogenous WT1 expression may delete or render anergic most of the high avidity T cell repertoire capable of recognizing WT1 expressing tumors. We hypothesized that *in vitro* engineering to increase TCR affinity would improve tumor cell recognition, although the potential for such high affinity TCRs to recognize normal tissues expressing low levels of WT1 would need to be assessed.

To test this hypothesis, we characterized the murine and human T cell responses elicited to $WT1_{RMFPNAPYL}$. Mice immunized with this peptide generate a diverse T cell response, recruiting T cells of multiple TCR Vβ families and encompassing an avidity range of three logs. However, none of the T cells isolated recognized murine tumors endogenously expressing WT1. We also isolated two human T cell clones specific for $WT1_{RMFPNAPYL}$, one of which recognizes human tumors expressing this antigen. The TCRs from these T cells are being used as templates for *in vitro* mutagenesis and selection of higher affinity TCRs by yeast display to determine the threshold required for efficient tumor recognition without targeting normal tissues.

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LIST OF ABBREVIATIONS

AML - acute myeloid leukemia

CDR - complementarity determining region

CML - chronic myeloid leukemia

CMV - cytomegalovirus

Cr - chromium

CTL - cytotoxic T lymphocyte

DLI - donor lymphocyte infusion

EBV - Epstein-Barr Virus

F2A - self-cleaving 2A peptide sequence derived from the foot and mouth disease virus

FBL - an FMuLV transformed murine erythroleukemia

FMuLV - Friend Murine Leukemia Virus

Gy - gray

GVHD - graft versus host disease

GVL - graft versus leukemia

HLA - human leukocyte antigen

HSCT - hematopoietic stem cell transplant

IFA - incomplete Freund's adjuvant

IFN-γ - interferon gamma

IL-2 - interleukin 2

IRES - internal ribosomal entry sequence

LCMV - lymphocytic choriomeningitis virus

LTR - long terminal repeat

mAb - monoclonal antibody

MFI - mean fluorescence intensity

MHC - major histocompatibility complex

MoMuLV - Moloney murine leukemia virus

OVA - ovalbumin

P14 - TCR transgenic mouse with a high frequency of naïve T cells specific for the KAVYNFATM peptide of LCMV

PBMC - peripheral blood mononuclear cells

Pgk - phosphoglycerate kinase

RT-PCR - reverse transcription polymerase chain reaction

TCR Vα/β - T cell receptor variable alpha or beta chain region

$TCR_{\alpha gag}$ - TCR transgenic mouse with a high frequency of naïve T cells specific for the CCLCLTVFL peptide of FMuLV

TAA - tumor associated antigen

TIL - tumor infiltrating lymphocytes

WT1 - Wilms' tumor antigen 1

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A number of people have helped to make this work possible. First and foremost, I'd like to thank my thesis advisor, Phil Greenberg. His enthusiasm for science and belief in the possible has been incredibly inspiring and helpful during the many times when my projects seemed to encounter challenge after challenge. I've been continually impressed that despite a busy travel schedule and splitting time between two sites, Phil consistently makes time for his students and post-docs, both meeting with them in person and providing detailed feedback on proposals and papers. From his written and oral comments over the years, I feel that I have learned a great deal about both evaluating and effectively communicating science. It has truly been a privilege and a joy to work in his lab.

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regarding the murine aspects of my projects over the years. I'd also like to thank Dr. Ryan Teague for helpful discussions and Xiaoxia Tan and Maria Huang for maintaining the mouse colony and for assistance with tumor therapy experiments.

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Chapter 1: Introduction and Background

Introduction:

Adoptive T cell immunotherapy has shown promise in treating human leukemias and malignant melanoma (1-4), however, the nature of tumor associated antigens, which are mostly derived from self-proteins, renders the generation of high avidity, tumor-reactive T cells difficult and has limited the widespread applicability of this technology to treat human cancers (5). To circumvent the challenges inherent in trying to generate tumor-reactive T cells *de novo* for each patient, T cell receptor (TCR) gene therapy, using retrovirus-mediated gene transfer of high affinity, tumor antigen-specific TCRs to impart desired specificities to patient's T cells, is being pursued (6, 7). However, this approach has also been limited, as few high affinity TCRs recognizing potential target antigens exist.

One promising tumor antigen target is the WT1 transcription factor. Compared to the low levels of expression found in a few normal adult tissues, it is overexpressed in most leukemias and many solid tumors, and expression contributes to the malignant phenotype (8-13). Data suggests that the WT1 protein is immunogenic, and T cell clones with sufficiently high avidity to selectively recognize tumors without harming normal tissues have been described (14-16). However, such T cells cannot be reproducibly isolated from the endogenous T cell repertoire, likely due to inherent tolerance to this self-protein.

We sought to isolate WT1-specific T cells from mice and humans and to use the encoded TCRs as templates to *in vitro* engineer higher affinity TCRs to determine if we could improve T cell recognition of WT1 expressing tumors and if there is an affinity threshold at which recognition of normal tissues occurs. The validation of such TCR modifications cannot be completed with *in vitro* testing, and we therefore desired to create a murine model to examine the functional properties of high affinity, WT1-specific TCR transduced T cells *in vivo* and to explore the possibility of using *in vitro* engineered, TCR transduced T cells targeting WT1 for adoptive T cell immunotherapy of humans.

We isolated and characterized both human and murine T cells specific for the WT1-derived peptide RMFPNAPYL. Although we isolated a human T cell clone that recognizes human tumors expressing high levels of WT1, we were unable to isolate murine T cells that similarly recognized WT1-expressing murine tumors. Efforts to generate higher affinity versions of the murine and human TCRs using yeast display are ongoing but have not yet yielded TCRs with the desired properties. Finally, to facilitate the testing of TCR gene therapy in tumor therapy models, we also examined optimization of retroviral vector design for expressing both chains of the TCR using a high affinity TCR specific for a model murine tumor antigen derived from $FMuLV_{gag}$.

Background:

T cells can contribute to tumor elimination, but endogenous responses are usually insufficient for tumor eradication.

Since the early 1900's the immune system, and T cells in particular, have been postulated to play an important role in recognizing and killing cancer cells (17). However, definitive evidence of the efficacy of T cells in tumor eradication has only come in recent decades. Studies of patients receiving hematopoietic stem cell transplants (HSCT) for hematologic malignancies revealed that individuals who develop both acute and chronic graft-versus-host disease (GVHD) have a lower risk of tumor relapse than patients who develop neither. Patients receiving grafts from genetically identical siblings, or T cell-depleted allografts, experience far less or no GVHD, but have a much higher risk of relapse than patients receiving HLA-matched but non-identical allografts or allografts not depleted of T cells (18). These data strongly suggest that allo-specific T cells can mediate a potent graft-versus-leukemia (GVL) effect. More recent studies have demonstrated that donor lymphocyte infusions (DLI) for patients who relapse following HSCT can induce complete remissions, particularly in patients with CML (19). The tissue distribution and expression levels of the minor histocompatibility allo-antigens recognized by donor T cells determine the targets of the GVL and GVHD effects, and many groups are working to identify lineage-specific minor histocompatibility antigens expressed by hematopoietic cells to maximize the GVL effect while avoiding the systemic toxicity associated with GVHD (20).

There is also increasing evidence that a fraction of cancer patients mount endogenous T cell responses to their tumors (21). Studies of patients with advanced ovarian or colorectal cancer have revealed that the presence of tumor infiltrating lymphocytes (TIL) is associated with significantly increased survival compared to patients without TIL, although the antigen-specificity of these T cells has not been defined (22, 23). Other studies using peptide-MHC tetramers have detected tumor antigen-specific T cells in the peripheral blood (24) and lymph nodes (25) of some patients, suggesting that strategies to augment endogenous T cell responses may promote tumor eradiation.

Nonetheless, attempts to boost endogenous T cell responses with cancer vaccines have been largely unsuccessful. A recent review examining the efficacy of several different types of cancer vaccines (peptide, pox virus, tumor cell, and dendritic cell-based), targeting a diverse array of tumor antigens, reveals that the overall clinical response rate is only ~3% (26). Unlike vaccines against infectious diseases, in which the target antigens are derived from foreign proteins, the majority of tumor-associated antigens (TAAs) are derived from non-mutated, self-proteins overexpressed by malignant cells (5). Central and peripheral tolerance mechanisms may delete or render anergic much of the high avidity repertoire that is most likely to effectively recognize and kill tumor cells. Furthermore, once patients have an established tumor, the high antigen load and generally immunosuppressive environment of the tumor makes it difficult to activate and expand *in vivo* any potential tumor-reactive repertoire that remains (5, 6).

Adoptive T cell immunotherapy overcomes the problems associated with in vivo expansion of tumor-specific T cells

An alternative approach to capitalize upon the therapeutic potential of T cells is to adoptively transfer large numbers of *in vitro* expanded, antigen-specific T cells into patients. Such adoptive T cell immunotherapy can overcome many of the obstacles that prevent the generation of an effective anti-tumor immune response *in vivo*, and the magnitude, specificity, avidity, and effector functions of the T cells infused into patients can be controlled (6, 27, 28). The efficacy of adoptive T cell immunotherapy for treating human diseases has been demonstrated with CMV (29, 30) and EBV (31) infections in HSCT recipients post-transplant, and promising results have been achieved in some patients with malignant melanoma (3, 4).

A critical factor in determining the success of adoptive T cell immunotherapy is the avidity of the infused T cells for the target antigen, as only high avidity T cells are capable of recognizing and lysing tumor cells (32, 33). Although T cell avidity for target cells is affected by several cell surface molecules, including costimulatory and adhesion molecules, the affinity of the T cell receptor (TCR) for peptide-MHC contributes the most to overall avidity. Thus, T cells bearing high affinity TCRs have a higher functional avidity, and recognize targets presenting lower amounts of antigen, than T cells bearing lower affinity TCRs (34). Due to self-tolerance mechanisms and/or tumor burden, the tumor antigen-specific T cells isolated from patients

frequently are of insufficient avidity to recognize and kill tumor cells and, consequently, are not directly useful for adoptive T cell immunotherapy.

T cell receptor gene therapy circumvents the limitation of having to generate high avidity T cells from every patient, but questions regarding maintenance of avidity in transduced T cells remain

TCR gene therapy has been suggested as a strategy to circumvent the biological and technical challenges inherent in trying to generate highly avid, tumor-specific T cells *de novo* for each patient (5-7, 35). The essence of T cell antigen recognition and reactivity resides in the TCR, and the genetic transfer of a TCR imparts recipient T cells with the ability to respond to the antigen recognized by the transferred TCR. In the setting of cancer therapy, patients could be screened for tumor antigen and MHC expression and their T cells retrovirally transduced with a high affinity TCR specific for the antigen and MHC allele their tumor expresses. Construction of a library of well-characterized, high affinity TCRs specific for a variety of tumor antigen-derived peptide MHC combinations would provide a panel of "off the shelf" reagents making adoptive T cell immunotherapy more broadly accessible to patients.

A number of TCRs recognizing human tumor antigens have been cloned, transferred via retroviral vectors to primary T cells, and demonstrated to impart tumor recognition to the transduced T cells (36-46). Translating this technology to patient care has become a high priority, as demonstrated by a recently published phase I

clinical trial in which T cells transduced with a MART-1-specific TCR were irradiated and injected intratumorally into patients with metastatic melanoma (47). No major toxicities resulting from the cells were reported, a partial response was observed in 1 of 15 patients, and 3 additional patients had temporary regression of a metastasis. Two additional trials of TCR gene therapy for melanoma are currently underway at the NCI (NCT00082264 and NCT00088439) and several others at academic institutions, including the University of Washington, are in advanced planning stages.

Although there is substantial impetus to move clinical trials of TCR gene therapy for cancer rapidly forward, basic questions regarding the magnitude and duration of transduced TCR expression and proper pairing of introduced TCRs on the cell surface remain. Studies examining polyclonal populations of freshly transduced T cells demonstrate wide variations in introduced TCR expression levels, and only cells with high surface levels exhibit avidities similar to those of the parental T cell clones from which the TCRs originated (48, 49). As surface expression of the introduced TCR decreases, avidity and antigen recognition rapidly decline. Recent studies have demonstrated that improvements in retroviral vector design (50, 51), elimination of cryptic splice sites within transgenes, and transgene codon optimization (52) can improve levels of TCR gene expression immediately after transduction and enhance antigen recognition in polyclonal T cell lines.

However, even when cells display high levels of transgene expression immediately after transduction, expression tends to decline with time. Most of the viral vectors currently used for TCR gene therapy are based upon a Moloney Murine

Leukemia Virus (MoMuLV) backbone and use the MoMuLV long-terminal repeat (LTR). Once integrated into host DNA, gene expression from these vectors can be lost over time due to silencing from methylation of the LTR (53, 54). Therefore, studies in animal models are necessary to assess modifications to vectors that promote maintenance of adequate TCR expression for a sufficient length of time to permit effective immunotherapy and tumor eradication.

Another concern regarding TCR gene therapy is the potential for mismatched pairing of the endogenous and introduced TCRα and β chains (7, 35). Such mismatched pairing not only decreases the number of appropriately matched transduced TCRαβ pairs at the cell surface, thereby decreasing T cell avidity for the target antigen, but also results in the formation of novel TCRαβ pairs that have not undergone thymic selection and could potentially lead to autoimmunity through self-antigen recognition. Strategies to preferentially promote pairing of the introduced TCRs with each other would not only enhance the therapeutic efficacy of adoptively transferred, TCR transduced T cells, but also improve the safety of such therapy.

The WT1 protein is a promising target for adoptive T cell therapy of leukemia and some solid tumors

Over the past decade, the Wilms' tumor antigen 1 (WT1) protein has received increasing attention as a promising target for T cell-based cancer immunotherapy. The WT1 protein is expressed in a variety of different isoforms and functions as both a transcription factor and a regulator of post-transcriptional processing (55-57). It was

named based on the observation that roughly 10% of Wilms' tumors, a pediatric kidney tumor, contain mutations or deletions of the gene encoding this protein. A number of different developmental urogenital abnormalities have also been associated with mutation or loss of function of the WT1 protein.

Although WT1 was originally characterized as a tumor suppressor, many studies have subsequently shown that, in a non-mutated, wild-type form, it is overexpressed in a variety of neoplasms, including most leukemias and many solid tumors, at 10-1000 fold higher levels than normal tissues (9-11). Furthermore, expression of WT1 is associated with maintenance of the tumorigenic phenotype, as down-regulation of WT1 expression with anti-sense oligonucleotides inhibits tumor cell proliferation and results in apoptosis (12, 13). Studies of leukemia patients suggest that overexpression of WT1 is associated with a worse prognosis and that the level of WT1 expression may be a useful prognostic marker (58-60). Expression of WT1 in normal human adult tissues is limited, but low levels have been detected in hematopoietic stem cells, podocytes of the kidney, sertoli cells of the testis, granulosa cells in the ovary, and mesothelial-derived cells lining the lung and spleen (8, 9).

In addition to being differentially expressed in tumor cells and normal tissues, a number of studies suggest that the WT1 protein is immunogenic (61, 62). T cells recognizing several MHC class I- (16, 41, 63-66) and class II- (67, 68) restricted epitopes from the WT1 protein have been isolated from healthy donors and leukemia patients. Clones derived from some of these responses lysed leukemic cells in an antigen-specific and HLA-restricted manner but did not inhibit the growth and

differentiation of normal hematopoietic progenitors *in vitro* (14-16). Furthermore, an HLA-A2-restricted, allogeneic $CD8^+$ T cell clone, or primary T cells transduced with the TCR from this clone, inhibited in a NOD/SCID murine model the engraftment of leukemia-initiating stem cells, but not normal hematopoietic progenitors (41, 69).

In addition, vaccination of patients with WT1-derived peptides has yielded promising results. It was recently reported that a patient with relapsed acute myeloid leukemia (AML) achieved a complete remission following vaccination with the HLA-A2-restricted WT1 peptide, RMFPNAPYL, with no evidence of autoimmunity detected (70). In another study, 26 patients with lung cancer, breast cancer, or leukemia were vaccinated with a HLA-A24 binding peptide (71). Clinical responses were detected in 12 patients, and the only observed toxicity was leukopenia in two patients with myelodysplastic syndrome, in whom all normal and abnormal hematopoiesis appeared to be derived from transformed stem cells overexpressing WT1.

Studies examining WT1 expression and immunogenicity in mice support the applicability of using this animal as a model for investigating T cell therapies targeting WT1. WT1 expression in both normal and malignant cells in mice mimics expression in human cells, and studies in murine models have already demonstrated the potential for targeting WT1 with immunotherapy (8, 61). Remarkably, the major $CD8^+$ T cell response to WT1 in C57BL/6 (B6) mice is also to the RMFPNAPYL peptide, but presented in the context of $H\text{-}2D^b$. Although HLA-A2 and $H\text{-}2D^b$ MHC molecules utilize different anchor residues within this peptide for presentation (M and L vs. N

and L; (72)), the conservation of epitope immunogenicity between species suggests a conserved peptide processing pathway. Mice immunized with the RMFPNAPYL peptide, or a plasmid encoding the entire WT1 protein, can generate $CD8^+$ T cells capable of killing WT1-expressing tumor cells (73-75). Furthermore, mice protected from challenge with WT1-expressing tumors by prior immunization do not show any signs of autoimmune damage to normal tissues that express low levels of WT1 (73, 75, 76).

Strategies to generate high affinity TCRs specific for self/tumor antigens

To examine the feasibility of using TCR gene therapy to target human tumors expressing WT1, as well as to create a murine model of adoptive T cell/TCR gene therapy targeting this antigen, we sought to isolate high avidity human and murine T cells specific for the WT1-derived epitope RMFPNAPYL that expressed high affinity TCRs for this antigen. However, as is the case with most self-antigens, isolating such T cells that can efficiently recognize tumors is difficult. Several different strategies have been described to isolate high affinity TCRs specific for self antigens that circumvent the challenges associated with a potentially deleted or anergized high avidity repertoire (5).

One such strategy is to isolate peptide- and MHC-specific T cells from the peripheral T cell repertoire of healthy, allogeneic individuals who do not express the restricting allele and thus have not been tolerized to the peptide-MHC complex. For example, $CD8^+$ T cells recognizing the WT1-derived RMFPNAPYL peptide in the

context of HLA-A2 have been generated from HLA-A2$^-$ donors (14, 15, 69). Studies of these T cells have provided important information with regard to immunological recognition of this target. However, it remains formally possible that such allo-restricted T cells may be more promiscuous than self-restricted T cells and recognize many other peptides bound to the same MHC allele. In fact, such broad cross-reactivity has been demonstrated for allogeneic T cells generated against the MART-1 antigen (77).

Another approach, demonstrated to permit isolation of high affinity MDM2 and p53-specific TCRs, is to immunize transgenic mice expressing human MHC molecules, such as HLA-A2, to generate high avidity T cells in a xenogeneic setting (78, 79). The resulting murine TCRs can subsequently be "humanized" which can diminish, though likely not completely eliminate, the immunogenicity of these xenogeneic proteins and consequent rejection by the patient's immune system. However, this approach also has additional limitations, since the presence of high avidity T cells in the mouse repertoire will be dependent on the mouse and human homologue differing in the particular epitope that binds to the transgenic human MHC allele, which will not be the case for many target proteins. Furthermore, the detection of high avidity T cells in the repertoire of mice may reflect differences in the selecting antigens in the thymus due to differences in the mouse and human proteomes - thus such xenogeneic TCRs, like allogeneic TCRs, could have unforeseen cross-reactivities when given to humans.

A third approach is to *in vitro* engineer higher affinity TCRs by sequential rounds of mutation and selection, using a low or moderate affinity tumor antigen specific TCR as a template. Several methods for selecting high affinity mutants from TCR libraries have been described, including bacteriophage expression (80, 81), yeast display (82, 83), and expression on the surface of a TCR deficient T cell line (84). For each of these strategies, large libraries must be generated and screened through several rounds of selection to identify higher affinity mutants. *In vitro* engineering can also be guided by a structural approach. The crystal structure of the low affinity TCR bound to peptide-MHC can be combined with computational modeling to suggest candidate residues for mutation to improve the binding energies of the intermolecular interactions (85-87). Theoretically, engineered, high-affinity TCRs could also exhibit broad cross-reactivity. However, high affinity mutants created through yeast (88, 89) and phage display (81) do not show signs of such cross-reactivity. In fact, it appears that these TCRs may be selected to be more specific for the targeted peptide-MHC complex based on reduced flexibility and an inability to assume alternative structural conformations that permit binding to other 'cross-reactive' peptide-MHC complexes (90).

While each of these approaches has inherent advantages and disadvantages, the prospect of creating a panel of high affinity TCRs specific for the same peptide-MHC is an appealing one. Analyzing and comparing the structural, biophysical, and biological properties of such a panel of receptors could yield valuable insights into how T cells recognize antigens and how the biophysical properties of a TCR translate

into biological responses. No analyses of this kind have been reported. In addition, we hypothesized that analysis of a panel of high affinity WT1-specific murine TCR mutants in an adoptive T cell immunotherapy model may allow the determination of how wide the window of differential recognition of tumor cells and normal cells can potentially be and how protective tolerance mechanisms are in eliminating the high avidity repertoire.

Based on available data when this project was initiated, we chose to use the yeast display method to *in vitro* engineer high affinity murine and human WT1-specific TCRs in collaboration with the Kranz lab (82, 88). They had demonstrated that TCRs can be mutated to increase the affinity by up to 1000 fold and that the resulting mutants were less cross-reactive than the wild-type TCR, exhibiting increased specificity for the peptide-MHC molecules they were selected on. While efforts to isolate high affinity, WT1-specific TCR mutants are still ongoing, analyses of the endogenous T cell responses elicited, and studies of TCR gene transfer, have yielded valuable insights for translating these ideas into human therapies.

Chapter 2: A Murine Model for T Cell Receptor Gene Therapy of Disseminated Leukemia

Introduction:

Prior to examining high affinity WT1-specific TCRs generated from *in vitro* mutagenesis and yeast display, we wanted to develop retroviral vectors permitting high levels of expression of both the transduced TCRα and TCRβ chains as well as techniques to model TCR gene therapy in the mouse. Our lab has previously designed a murine model for adoptive T cell immunotherapy of disseminated leukemia using T cells with a TCR specific for a Friend Murine Leukemia Virus- (FMuLV) derived gag epitope, expressed by the erythroleukemia FBL (91). We used this model to test if mice could be cured of FBL leukemia using T cells transduced with the gag-specific TCR and to determine how long transduced TCR expression could be maintained *in vivo*. We also asked if tumor antigen recognition by transduced T cells could be improved by stimulating the T cells through the endogenous TCR. Our results demonstrate that transduced TCR expression can be maintained on a subset of T cells for up to seven months *in vivo* and that activation of transduced T cells through the endogenous TCR can increase the number of transduced T cells capable of recognizing tumor antigen through the introduced TCR.

Materials and Methods:

Mice: C56BL/6 (B6) and Thy1.1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). P14 mice were a kind gift from Dr. Murali Krishna-Kaja and were

bred with Thy1.1 congenic mice to generate $P14^{+/-}$x$Thy1.1^{+/-}$ heterozygotes as a source of P14 $Thy1.1^{+}$ T cells. $TCR_{\alpha gag}$ mice were generated in our lab (92).

Peptides, antibodies, and media: $FMuLV_{gag}$ (CCLCLTVFL in which C is replaced by aminobutyric acid) and LCMV-gp33 (KAVYNFATM) –derived peptides were synthesized and HPLC purified by Synpep (Dublin, CA). Vα2, Vα3, Vβ8, Vβ12, CD8α, Thy1.1, IFN-γ, and anti- FcγIII/IIR antibodies were purchased from BD Pharmingen (San Diego, CA). All cell culture was performed using RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 25 mM HEPES, 2 μM L-glutamine, 100 U/mL penicillin/streptomycin, 10% fetal calf serum, and 30 μM 2-mercapatoethanol (complete medium).

Vector construction and virus preparation: The pLZRSpBMN-Z retroviral vector was a kind gift from Dr. Gary Nolan. The murine phosphoglycerate kinase (pgk) promoter was cloned from pMSCVpuro (Clontech, Palo Alto, CA), the IRES from pIRES2EGFP (Clontech), and the F2A sequence created using primers as described (93); TCRα and β chain genes were cloned from the cDNA used to create $TCR_{\alpha gag}$ TCR transgenic mice (92). To produce retroviral supernantant, pLZRS plasmids containing the Vα3 and Vβ12 TCR chains were transfected into Phoenix E packaging cells using Lipofectamine and selected with puromycin (1 μg/mL). $2x10^7$ selected packaging cells were plated without puromycin in T-225 flasks at 37°C and viral supernatant was harvested two days later. Fresh media was added to the flasks, and

after a 20 hour incubation at 32°C additional viral supernatant was harvested. To concentrate the virus, 10 mL of PEG solution (100 g polyethylene glycol MW 8000 + 6 g NaCl in 250 mL H_2O, pH 7.2) was added to 40 mL of virus supernatant, stored at 4°C for 1-2 days and centrifuged at 2500 rpm (~1500 x g) for 45 minutes at 4°C. Virus pellets were resuspended in 500 μL complete medium and used immediately for transduction or stored frozen at -80°C.

T cell transduction and in vitro cell culture protocol: $4x10^7$ splenocytes from P14xThy1.1 mice were stimulated *in vitro* with gp33 peptide (3 μM final concentration) and 20 U/mL IL-2 in 12 mL complete medium in T-25 flasks (upright) for 25 hours. Cells were washed and resuspended at $1x10^8$ cells per mL. 50 μL of cells were spinfected in a table top centrifuge in 6 well plates with 1.5 mL concentrated retroviral supernatant, IL-2 (20 U/mL), and polybrene (2 ug/mL) at 1500 rpm (470xg) for 1 hour at 32°C then incubated at 37°C for 16 hrs. Cells were washed and resuspended in fresh medium with IL-2 (20 U/mL) and were restimulated with antigen every 9-10 days. Mock transduced P14 T cells were restimulated with $5x10^6$ irradiated (3000 rads) B6 splenocytes as feeders in 12 mL complete medium with 0.16 μg/mL gp33 peptide and 20 U/mL IL-2. $TCR_{\alpha gag}$ transgenic T cells and P14 T cells transduced with the Vα3Vβ12 retroviruses were stimulated with $5x10^6$ irradiated B6 feeders, $2x10^6$ irradiated (10,000 rads) FBL, and 20 U/mL IL-2 in 12 mL complete medium.

Tumor therapy protocol: Adoptive immunotherapy was performed as previously described (91). Briefly B6 mice received $2x10^6$ live FBL tumor cells from fresh ascites i.p. Five days later when tumor was widely disseminated mice received 180 mg/kg cyclophosphamide i.p. and six hours later $6x10^6$ T cells i.v. On days 5-15 mice received $1x10^4$ U IL-2 i.p. Mice were monitored for ascites formation and were euthanized when a detectable tumor burden increased to size that predictably led to morbidity/mortality within 24-48 hours and/or the mice exhibited severe tumor-associated morbidity.

In vitro stimulation and intracellular cytokine staining: 20-30$x10^6$ splenocytes were stimulated in T-25 flasks with 20 U/mL IL-2, $5x10^6$ irradiated (10,000 rads) FBL or 0.04 μg/mL gp33 peptide in 12 mL complete media for 6 days. $1x10^6$ live cells from each of these cultures were stimulated a second time with gag, gp33, or no peptide (2.5 μg/mL) in 96 well plates with 10 U/mL IL-2 for 6 hours, in the presence of golgi-plug (BD Pharmingen) for the last 5 hours. Cells were incubated with antibodies to CD8α and Thy1.1, fixed and permeabilized (cytofix/cytoperm, BD Pharmingen), washed (perm/wash, BD Pharmingen), incubated with antibodies to IFN-γ, and visualized on a flow cytometer.

Results:

In a well-characterized model of adoptive T cell immunotherapy for leukemia, $TCR_{\alpha gag}$ T cells eliminate disseminated tumor and permit survival of recipient mice

(91, 92). We used this model to ask several questions relevant to designing TCR gene therapy protocols: a) how should TCRα and β chains be arranged within a single retroviral vector to achieve the highest levels of expression of the introduced TCR genes on the surface of T cells, b) how long can expression of transduced TCR genes be maintained *in vivo* on persisting T cells, and c) can T cell responses to the antigen recognized by the transduced TCR be enhanced by first stimulating transduced T cells through the endogenous TCR.

To answer the first question, the cDNAs encoding the TCRα (Vα3) and β (Vβ12) chains expressed by $TCR_{\alpha gag}$ T cells were cloned into three different retroviral vectors to create the viral constructs shown in Figure 1. All three vectors use the same MoMuLV LTRs and backbone. The TCRα and β chains inserted into these vectors were separated either by a murine phosphoglycerate kinase (pgk) promoter, an IRES element, or the self-cleaving F2A peptide derived from the foot-and-mouth disease virus that was previously used to create multicistronic vectors expressing equivalent levels of TCRα and β chain genes (93). The Vα3Vβ12 TCR recognizes an epitope within the gag protein of FMuLV expressed by the murine erythroleukemia FBL. Retroviruses encoding these 3 different versions of the gag-specific TCR were transduced into T cells from P14 TCR transgenic mice expressing an endogenous Vα2Vβ8 TCR recognizing an epitope from the gp33 protein of lymphocytic choriomeningitis virus (LCMV). Transduced and mock transduced T cells were incubated with antibodies to Vα3 and Vβ12 to assess transduction efficiencies and levels of transduced TCR expression. Transduction efficiencies ranged from 25% to

60%. Although the levels of Vα3 and Vβ12 expression appeared equivalent in T cells transduced with the IRES and F2A containing retroviruses, expression of the Vβ12 chain was substantially weaker in T cells transduced with the pgk promoter construct (Figure 2). Because the latter cells proliferated poorly *in vitro* when stimulated with irradiated FBL, we decided not to expand them to numbers sufficient for *in vivo* therapy and only used T cells transduced with the IRES and F2A containing vectors for adoptive immunotherapy.

Prior to infusion into tumor bearing mice, $TCR_{\alpha gag}$ transduced and mock transduced P14 T cells were expanded *in vitro* for multiple cycles to simulate protocols used for adoptive T cell therapy in humans. It generally requires two to three months of *in vitro* culture to isolate and expand a T cell clone to yield sufficient numbers of cells for patient infusions, and continued expansion *in vitro* can eventually lead to terminal differentiation and loss of proliferative potential (6). After eight stimulation cycles (~2.5 months in culture) the Vα3IRESVβ12 transduced T cell population maintained expression of both the introduced and endogenous TCRs at high levels, such that greater than 90% of the cells expressed all four TCR chains (Figure 3a,b). However, by the thirteenth stimulation cycle, some of the Vα3IRESVβ12 cells had lost expression of the Vα3 TCR chain, such that only 76% of the T cells expressed high levels of both Vα3 and Vβ12 (Figure 3c). For the Vα3F2AVβ12 transduced T cells, high levels of the introduced TCR were found on 77% of cells and high levels of the endogenous TCR on 85% of the cells after eight

cycles of *in vitro* stimulation. Levels of the introduced TCR remained similar even after the thirteenth *in vitro* stimulation (Figure 3).

To test whether these Vα3Vβ12 TCR transduced T cells could cure mice of disseminated FBL tumor, $6x10^6$ mock or TCR transduced T cells were injected into B6 mice five days after tumor inoculation. The T cell lines had been stimulated *in vitro* nine times since initially activated for retroviral transduction. In addition, one group of mice received T cells from an *in vitro* maintained T cell line generated from $TCR_{\alpha gag}$ transgenic mice as a positive control for tumor elimination. In this model of tumor immunotherapy, mice receiving cyclophosphamide and IL-2 alone, or in combination with non-specific T cells, die within 3 to 4 weeks of tumor inoculation (94), and antigen-specific T cells must persist for at least 30 days to completely eliminate tumor (95). Four of four mice that received the *in vitro* cultured T cell line derived from $TCR_{\alpha gag}$ transgenic mice rejected their tumors and survived disease-free for over 150 days (Figure 4). Similarly, five of five mice that received Vα3IRESVβ12 transduced P14 T cells rejected tumor and survived. Of the five mice that received Vα3F2AVβ12 transduced P14 T cells, three succumbed to tumor on day 25, with kinetics similar to the mice that received mock transduced P14 T cells, while two mice rejected tumor and survived long-term.

On day 214 after tumor challenge (or 295 days from the time the P14 T cells were initially activated for retroviral transduction), surviving mice were euthanized and splenocytes stimulated *in vitro* with either irradiated FBL or gp33 peptide. Six days later, all cells were restimulated with either no peptide, gag peptide, or gp33

peptide in the presence of brefeldin A for six hours, and analyzed for IFN-γ production by flow cytometry to determine if the transduced T cells persisted, if they retained sufficient expression of the introduced TCR to proliferate in response to tumor, and if tumor antigen recognition could be enhanced by first stimulating the transduced T cells through the endogenous TCR.

Transduced P14 T cells expressed the congenic marker Thy1.1 and were detected in five of the six surviving mice (one Vα3IRESVβ12 recipient died of unknown causes on day 197). The frequency of responding P14 T cells following primary FBL or gp33 stimulation varied greatly between mice. In four of five mice, FBL stimulation failed to appreciably expand transduced T cells, such that less than 0.3% of the $CD8^+$ T cells were of P14 origin (Figure 5 and data not shown), suggesting that most of the transduced T cells in these mice had down-regulated expression of the gag-specific TCR. Compared to stimulation with FBL, gp33 peptide stimulation resulted in 10-70 fold greater numbers of P14 T cells. The dramatic expansion of these cells in response to gp33 resulted in greater numbers (and a somewhat greater proportion) of T cells capable of producing IFN-γ in response to gag peptide restimulation, suggesting that stimulation of transduced T cells through the endogenous TCR may be one strategy to increase the number of T cells that can recognize tumor cells *in vivo*.

Unexpectedly, in one of the five mice, ~9% of the $CD8^+$ T cells were of P14 origin following FBL stimulation (~25% following gp33 stimulation), suggesting that in this mouse, the majority of transduced T cells retained expression of the gag-

specific TCR. However, on gag peptide restimulation, only half of these T cells produced IFN-γ, compared to 90% of cells restimulated with gp33 peptide, indicating that, in this six hour assay, the gp33 peptide delivers an intrinsically better signal. Thus, our results may underestimate the number of T cells capable of functionally responding to the gag antigen.

Discussion:

In this model of TCR gene therapy, P14 T cells transduced with a Vα3Vβ12 TCR specific for the gag antigen expressed by FBL effectively mediate tumor rejection and survival of mice with disseminated leukemia. Our results yielded several insights regarding retroviral vector design and TCR gene transduction for adoptive immunotherapy.

First, the IRES and F2A containing vectors expressed roughly equivalent levels of introduced TCRα and β chains both immediately post transduction and over the four month period the transduced T cells were maintained *in vitro*. However, expression of the Vβ12 TCR chain driven by an internal pgk promoter in the third construct was substantially reduced compared to the other two constructs. Vβ12 expression in these T cells did not improve in response to FBL stimulation in culture, suggesting it was difficult to select even a subpopulation with strong expression of Vβ12. In fact, these T cells did not expand well *in vitro*, indicating that the reduced TCR expression resulted in reduced capacity to recognize and respond to antigen. Two previous reports have suggested that the pgk promoter yields gene expression

levels equivalent to IRES-containing vectors (37, 39), and one other report demonstrated only slightly reduced levels of expression (96). Nonetheless, when persistent expression was examined, the pgk promoter yielded significantly reduced levels of gene expression compared to the MoMuLV LTR (50).

Effective tumor immunotherapy also requires T cell persistence and maintenance of T cell activity *in vivo*. All five of the mice that received Vα3IRESVβ12 transduced P14 T cells rejected tumor and survived, however, it is unclear why only two of the five mice that received Vα3F2AVβ12 transduced P14 T cells survived. Although we cannot definitively conclude that IRES containing vectors are superior to F2A containing vectors in this model with this limited data set, it is possible that some of the mice rejected the Vα3F2AVβ12 transduced P14 T cells. The F2A peptide contains a probable H2-D^b binding epitope (QTLNFDLLK; (72)) and adoptively transferred T cells can serve as potent APCs for the generation of immune responses (97). Whether this F2A-derived peptide is presented to host T cells and is immunogenic is currently being explored.

Adoptively transferred T cells were recovered from five of the six mice that received transduced P14 T cells and survived to day 214. Of the few published murine tumor immunotherapy studies that demonstrate the effectiveness of TCR gene transfer to mature peripheral T cells (98-101), only one has demonstrated maintenance of transduced T cells in an immunocompetent mouse (101). These T cells proliferated in response to tumor rechallenge three months following the initial tumor challenge. Our results extend these observations, demonstrating that transduced T cells can be

maintained in most mice for up to seven months, but that the majority of these T cells loose expression of the transduced TCR and cannot respond to the tumor antigen at this time.

One strategy that has been proposed to help promote persistence of TCR transduced T cells *in vivo* is to transduce tumor-specific TCRs into T cells expressing endogenous receptors specific for latent viruses such as CMV and EBV (102). Some of these virus-specific T cells exhibit a memory phenotype and should be long-lived. In addition, the periodic endogenous viral reactivation that occurs in healthy individuals might help maintain this pool of T cells by intermittently triggering T cell proliferation.

In our model, the transduced T cells expressed an endogenous TCR specific for a viral antigen derived from gp33 of LCMV. When we stimulated T cells from surviving mice *in vitro*, we observed a 10-70 fold greater T cell proliferation in response to gp33 peptide compared to FBL tumor, likely due to down-regulation of the gag-specific TCR in many of the transduced T cells. Expression of retrovirally encoded genes is frequently down-regulated in quiescent T cells, but can be enhanced following cellular activation (103). Both the number and proportion of transduced T cells capable of producing IFN-γ in response to gag peptide restimulation was higher in cells initially stimulated with gp33 compared to cells initially stimulated with irradiated FBL tumor. These results suggest another potential advantage to transducing tumor-specific TCRs into T cells specific for known, or latent viral, antigens. The periodic stimulation these T cells receive *in vivo* may not only help to

maintain T cell numbers, but may also enhance expression of the transduced TCR, and increase the total number of T cells capable of responding to residual tumor cells.

Additional data from our lab suggests a third reason why transducing tumor-specific TCRs into T cells specific for latent viral antigens, or another known foreign antigen, may be advantageous for cancer immunotherapy. TCR transgenic mice expressing the gp33-specific P14 TCR were bred with $TCR_{\alpha gag}$ mice expressing the gag-specific TCR. $CD8^+$ T cells isolated from the resulting transgenic mice endogenously express both TCRs and proliferate in response to both antigens. However, if these two-TCR mice are bred with another transgenic mouse that expresses the gag antigen in the liver, $CD8^+$ T cells from these triple transgenic mice proliferate in response to gp33, but not in response to gag peptide. In this model T cell tolerance is regulated at the level of the TCR that encounters the tolerizing signal, and not more globally at the cellular level (Teague, et. al., manuscript in preparation). Moreover, tolerance to the gag antigen can be broken by stimulating T cell proliferation through activation of the gp33-specific TCR. This data suggests that if chronic exposure to tumor antigen *in vivo* renders adoptively transferred, TCR transduced T cells tolerant, one may be able to rescue these cells and enable them to respond to tumor antigen again if they are stimulated through the endogenous receptor.

Although long-term maintenance of transduced TCR expression was observed in adoptively transfered T cells in one mouse, most transduced T cells in the other mice had down-regulated expression of the Vα3Vβ12 TCR. The retroviral vector used for these studies is a relatively early generation MoMuLV vector, and a number

of improvements in vector design to enhance transgene expression are possible (104). The incorporation of alternative promoters (50, 105), insulator sequences (51), scaffold attachment regions (50, 53), and the post-transcriptional regulatory element from the woodchuck hepatitis virus (105) have all been shown to enhance transgene expression in quiescent cells and/or prevent integration site methylation. Hence, with relatively simple vector improvements, incorporating one or more of these elements, it may be possible to maintain TCR transgene expression at high levels for longer durations. Additional studies examining some of these strategies are underway.

In summary, we demonstrate that transduction of virus-specific murine T cells with a tumor antigen-specific TCR cures mice of disseminated tumor. Although retroviral vectors permitting high levels of expression of both the TCRα and β chains within the same construct were identified, TCR expression was maintained on only a fraction of cells after seven months *in vivo*. Finally, the transduction of tumor antigen-specific TCRs into virus-specific T cells (or simply T cells with a known antigen specificity) represents a possible strategy to enhance both the *in vivo* persistence of adoptively transferred T cells as well as the maintenance of transduced TCR expression and the ability of these T cells to recognize tumor cells *in vivo*.

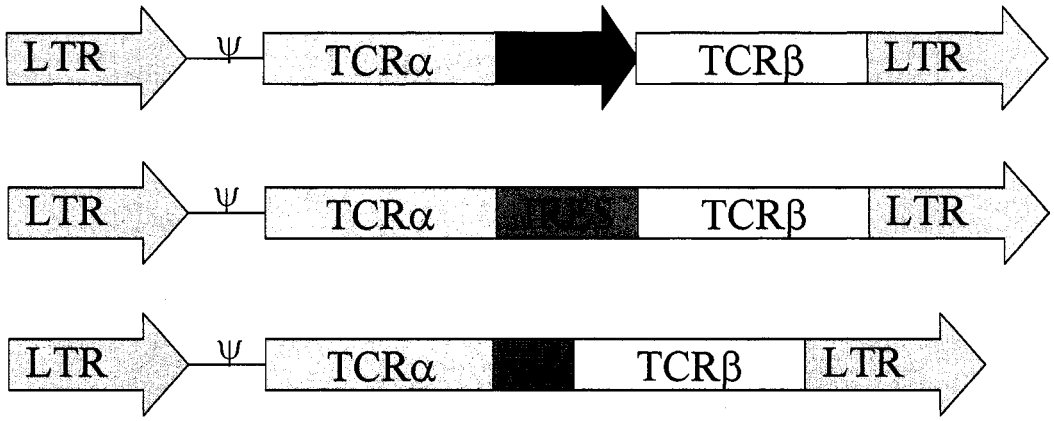

Figure 1: Design of retroviral constructs. $TCR_{\alpha gag}$ TCRα and β chains were cloned into pUC6's' and separated by an IRES element, murine pgk promoter, or F2A sequence and the entire cassette cloned into the BamHI and NotI sites of the retroviral vector pLZRSpBMN-Z, replacing the lacZ gene. Only the retrovirus portion of the vector is shown.

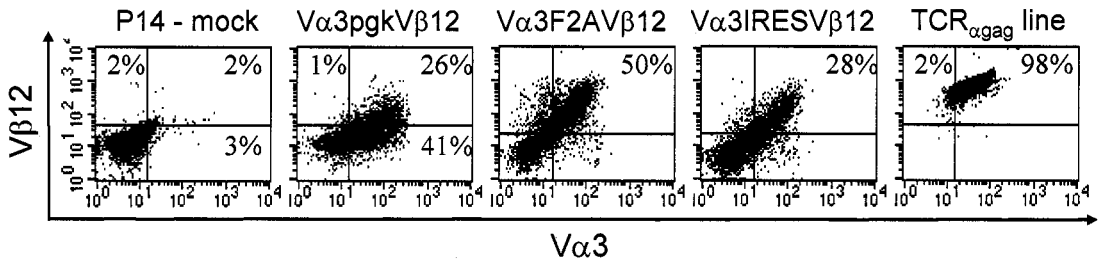

Figure 2: $TCR_{\alpha gag}$ TCRα and β chain expression in P14 T cells. P14 T cells were transduced with one of the 3 retrovirus constructs shown in Figure 1 and stained with antibodies to CD8α and the Vα3 and Vβ12 TCR chains specific for the gag antigen 5 or 6 days post-transduction. Plots shown are gated on $CD8^+$ lymphocytes. Staining controls include mock transduced P14 T cells as shown on the far left and a $TCR_{\alpha gag}$ T cell line on the far right.

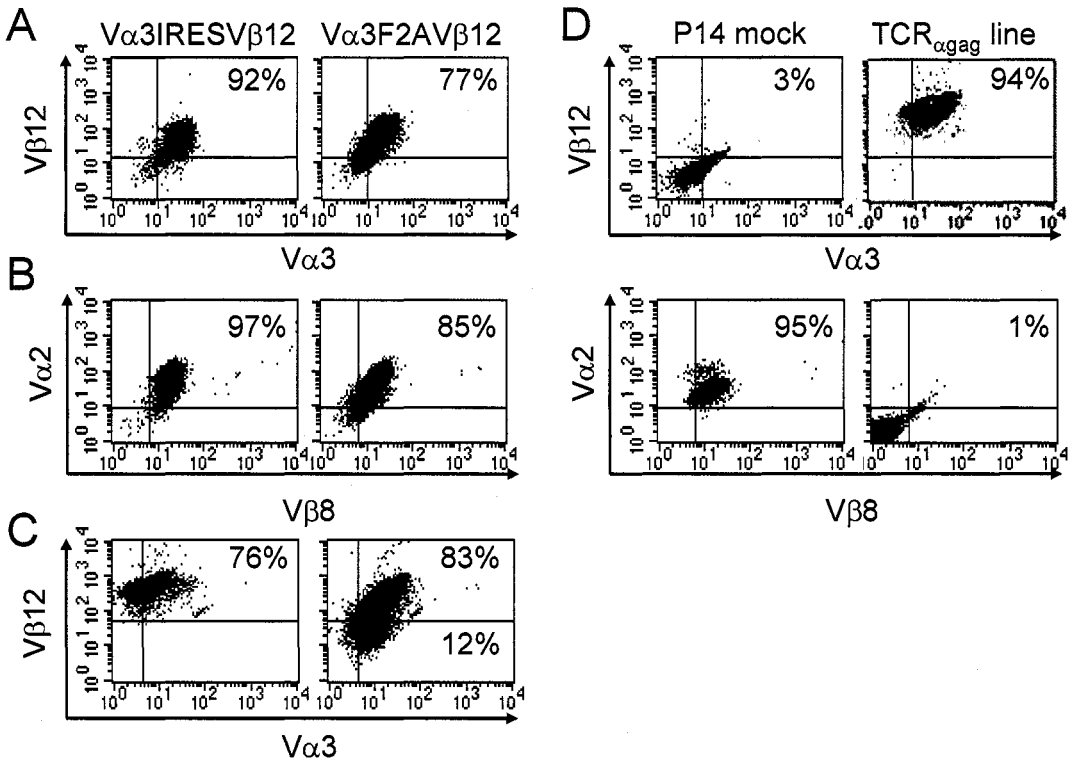

Figure 3: Transduced TCR expression is maintained *in vitro* over time. $TCR_{\alpha gag}$ TCR transduced P14 T cells were stained with antibodies to CD8α and the introduced gag-specific TCR chains (Vα3 and Vβ12) or the endogenous gp33-specific TCR chains (Vα2 and Vβ8). A) Expression of the gag-specific TCR on day 9 following the 8th *in vitro* stimulation when cells had been in culture for ~2.5 months. B) Expression of the gp33-specific TCR at the same time point as in A. C) Expression of the gag-specific TCR 9 days following the 13th *in vitro* stimulation. D) Staining controls include mock transduced P14 T cells and a $TCR_{\alpha gag}$ T cell line. All plots shown are gated on $CD8^+$ lymphocytes.

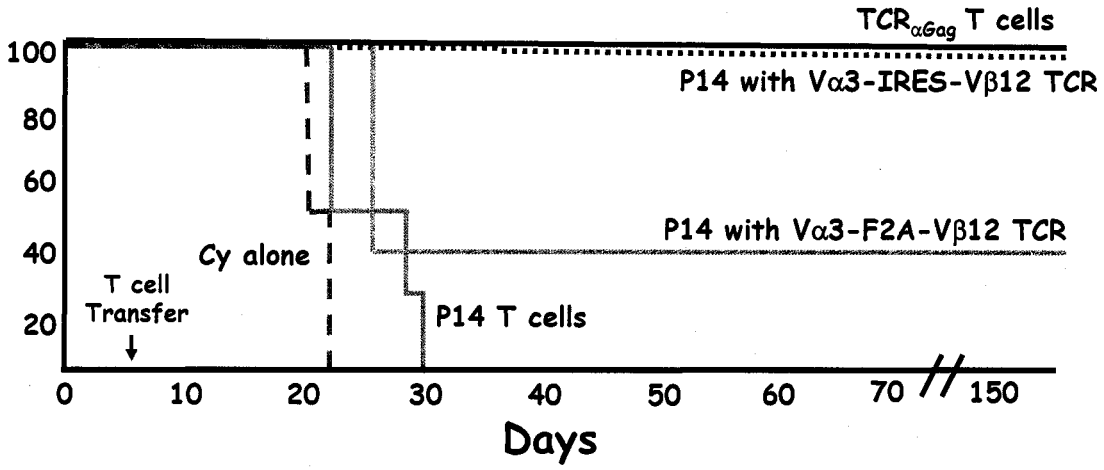

Figure 4: P14 T cells transduced with a gag-specific TCR can cure mice of disseminated tumor. B6 mice were injected with $2x10^6$ live FBL tumor cells i.p. on day 0 and cyclophosphamide (Cy) on day 5. Six hours following Cy treatment mice received no T cells (dashed tourqoise line) or $6x10^6$ mock transduced P14 T cells (solid green line), P14 T cells transduced with a Vα3F2AVβ12 retrovirus (solid orange line), P14 T cells transduced with a Vα3IRESVβ12 retrovirus (dotted red line), or $TCR_{\alpha gag}$ T cells (solid dark blue line). Mice were euthanized when tumors became visible.

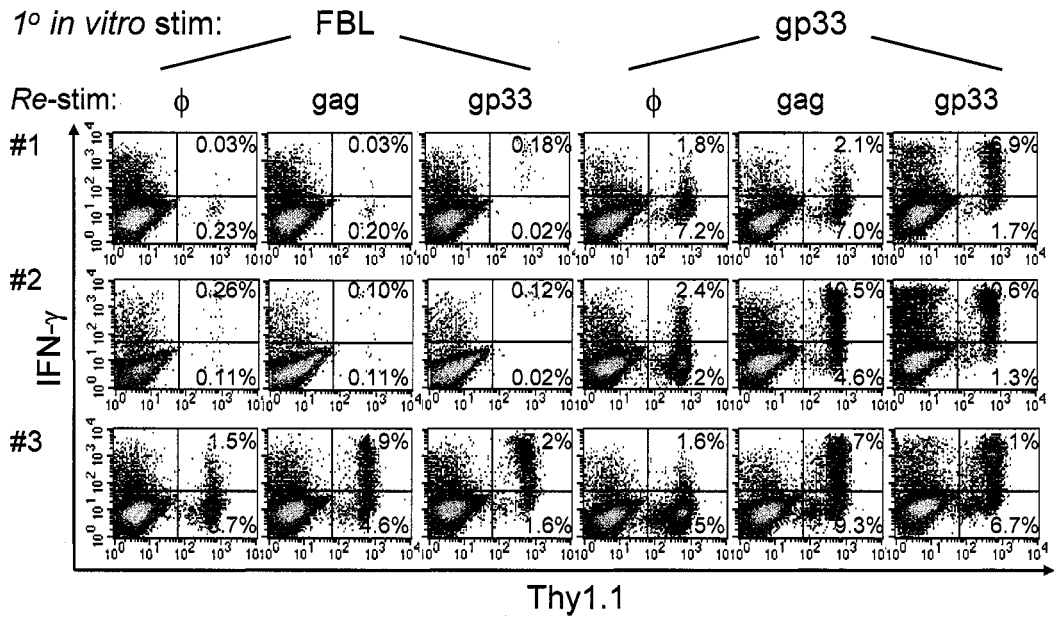

Figure 5: Long-term maintenance and expansion of tumor-specific T cells. On day 214 following tumor challenge, splenocytes from surviving mice were stimulated *in vitro* with irradiated FBL or gp33 peptide. Six days later, cells were restimulated with no peptide (ϕ), gag peptide, or gp33 peptide for 6 hours and analyzed for production of IFN-γ. Results from three representative mice are shown. Mouse #1 received Vα3F2AVβ12 transduced P14 T cells; mice #2 and #3 received Vα3IRESVβ12 transduced T cells. The majority of transduced T cells in mouse #3 retained expression of the gag-specific TCR. All plots are gated on $CD8^+$ cells. Transduced and adoptively transferred P14 T cells were congenically marked with Thy1.1.

Chapter 3: Isolation and Characterization of Murine WT1-Specific T Cells

Introduction:

To create a murine model for adoptive T cell immunotherapy targeting the WT1 antigen, we sought to generate murine $CD8^+$ T cells specific for the RMFPNAPYL epitope with the capacity to lyse murine tumors endogenously expressing WT1. As tolerance mechanisms operative for this self-protein may limit the avidity of T cells available from the endogenous repertoire, we examined a variety of immunization regimens to determine the highest avidity response to this epitope that could be elicited. The TCR variable domains used by the T cells responding to this WT1 epitope were characterized to gain insights into the breadth of response to this epitope and to identify potential TCRs for use as templates to *in vitro* engineer higher affinity RMFPNAPYL-specific TCRs. WT1 expression in several murine tumors and murine thymus was also analyzed to better understand the relationship between the amount of antigen that will be expressed by potential therapeutic targets and the amount of antigen that may be encountered by T cells during development.

Materials and Methods:

Mice: C56BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Peptides, antibodies, & tetramer: WT1 (RMFPNAPYL), ovalbumin (SIINFEKL), $FMuLV_{gag}$ (CCLCLTVFL in which C is replaced by aminobutyric acid), $FMuLV_{env}$ (EPLTSLTPRCNTAWNRLKL), and $LCMV_{gp33}$ (KAVYNFATM) – derived peptides were synthesized and HPLC purified by Synpep (Dublin, CA). Vα2, Vα3, Vα11, Vβ5, Vβ7, Vβ8, Vβ12, Vβ13, Vβ14, CD8α, IFN-γ, and anti-FcγIII/IIR antibodies were purchased from BD Pharmingen (San Diego, CA). The αCD3 and αCD28 mAb were a kind gift from Dr. Jeffrey Bluestone. The PE-labeled RMFPNAPYL-H2-D^b tetramer was purchased from Immunomics (Beckman Coulter, San Diego, CA).

Immunizations: Clone #33 was generated from a mouse immunized twice each with 25 μg WT1-derived RMFPNAPYL peptide and 50 μg of the class II-restricted FMuLV envelope-derived EPLTSLTPRCNTAWNRLKL peptide emulsified in 75 uL of incomplete Freund's adjuvant administered s.c. The M2 line was generated from a mouse primed with ~ $5x10^7$ irradiated (30 Gy), RMFPNAPYL peptide-pulsed (6 μg/mL for 60 minutes) adherent splenocytes activated for 24-36 hours with LPS (10 μg/mL) and αCD40 antibody (FGK45 at 5 μg/mL). Four weeks following priming, mice were boosted with whole splenocytes activated and pulsed in the same manner. Lines 1A, 2A, 3C, and 3D originated from mice primed with activated, RMFPNAPYL peptide-pulsed adherent splenocytes and boosted with a first generation recombinant Listeria encoding the RMFPNAPYL epitope within a piece of the ovalbumin gene fused to the LLO protein coding sequence. The mice from which lines 1A and 2A originated were euthanized at the peak of the Listeria response. Mouse #3 (the source

of lines 3C and 3D) was maintained in the mouse colony for several months following Listeria infection until it had to be euthanized following the development of dermatitis. Line #5770C was generated from a mouse immunized twice with WT1 and $FMuLV_{env}$-peptide pulsed, irradiated, LPS-activated (no αCD40) whole splenocytes and boosted with these same peptides emulsified in IFA.

In vitro T cell culture: All cell culture was performed using RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 25 mM HEPES, 2 μM L-glutamine, 100 U/mL penicillin/streptomycin, 10% fetal calf serum, and 30 μM 2-mercapatoethanol (complete medium). To generate WT1-specific T cell lines, $20\text{-}30x10^6$ splenocytes were stimulated in T-25 flasks with 20 U/mL IL-2 and 0.1-2.5 μg/mL RMFPNAPYL peptide in 12 mL complete medium. Lines were restimulated every 9-10 days with $5x10^6$ irradiated (30 Gy) B6 splenocytes as feeders, 0.1-2.5 μg/mL RMFPNAPYL peptide, and 20 U/mL IL-2 in 12 mL complete medium and split as needed

Cell lines: T2 cells transfected with $H2\text{-}D^b$, K^b, and L^d were a kind gift from Dr. David Kranz. FBL is a FMuLV-transformed erythroleukemia and E10 a FMuLV-negative derivative of the EL-4 thymoma line. The TRAMP-C1A prostate tumor line was a kind gift from Dr. Norman Greenberg, the SV40 transformed fibroblast line BLKSV40 and its untransformed parental line BLKCL4 were gifts from Dr. Martin Cheever, and B6 thymus RNA was provided by Dr. Andrew Farr.

Listeria: All *Listeria monocytogenes* vectors were created and provided by Cerus Corporation and contained OVA fusion proteins inserted into the $tRNA^{Arg}$ locus of the *Listeria monocytogenes* chromosome in an attenuated actA- and inlB-deficient strain. The first generation WT1-Listeria vector contained an LLO-OVA fusion protein driven by the hly promoter with the RMFPNAPYL epitope positioned immediately downstream of the SIINFEKL epitope. The second generation WT1-Listeria vector contained an ActA-OVA fusion protein driven by the actA promoter with the RMFPNAPYL epitope positioned at the junction of the ActA and OVA fusion.

TCR identification and cloning: The TCR chains expressed by clone #33 were amplified using a SMART RACE cDNA Amplification Kit (Clontech #K1811-1) and the following primers: TCA ACT GGA CCA CAG CCT CAG CGT CA (Cα), GGC AGA ATT TGG GAT GCA CAG ACA A (Cβ1), and GCC AGG GTG AAG AAC GGC TCA GGA T (Cβ2). PCR products were gel purified, cloned into pCR2.1 TOPO, sequenced, and BLAST searched using NCBI.

Vector construction and virus preparation: The pLZRSpBMN-Z retroviral vector was a kind gift from Dr. Gary Nolan. The clone #33 Vα3 and Vβ11 TCR chains were individually cloned into the BamHI and NotI sites removing the LacZ gene to form pLZRSVα3 and pLZRSVβ11. Retroviral vectors were transfected into Phoenix E packaging cells using Lipofectamine and selected with puromycin (1 μg/mL). Selected packaging cells were plated at 50% confluency without puromycin in T-75

flasks in 7.5 mL of complete media. Viral supernatant was harvested 2 days later and stored on ice. Packaging cells were treated with 50 μg/ml mitomycin C for 30 minutes, rinsed twice with PBS, incubated for an additional hour at 37°C, and trypsinized. Equivalent numbers of Vα3 and Vβ12 producing packaging cells were mixed and resuspended in the original viral supernatant ($0.5x10^6$ cells / mL).

T cell transduction: Six well plates were coated with αCD3 and αCD28 antibodies (1 μg/mL each, 1 mL/well) overnight at 4°C. Splenocytes from P14xThy1.1 mice were washed and resuspended at $5x10^6$ lymphocytes/mL and 1 mL added to each well. After a 25 hour incubation at 37°C, 2-3 mL of mitomycin C treated packaging cells resuspended in retroviral supernatant was added to each well along with IL-2 (20 U/mL) and polybrene (2 ug/mL). Splenocytes were spin-infected at 1500 rpm (470xg) for 1 hour at 32°C then incubated at 37°C. After 16 hours fresh media and IL-2 were added.

Chromium release assays: $1.5x10^6$ target cells were radioactively labeled with 100 μCi ^{51}Cr at 37°C for 60 minutes in 50 μL complete medium. Following three washes, $4\text{-}5x10^3$ cells were plated per well of a 96 well plate. Cells were pulsed with ~ 6 μg/mL of the appropriate peptide during the chromium incubation, or the indicated amount of peptide was added directly to the well for the peptide titration assays. T cells were added at the indicated effector to target cell ratios and all effector-target combinations were assayed in triplicate. Total volume per well was 200 μL. After a 4

hour incubation at 37°C, 70 μL of supernatant was removed from each well and counted in a gamma counter. Percent specific lysis was calculated as: (average sample lysis – average spontaneous lysis)/(average total lysis – average spontaneous lysis)x100.

Assay for intracellular IFN-γ production: 1-3x10^6 splenocytes or cells from *in vitro* cultured lines were incubated with the appropriate peptide (2.5 μg/mL) and IL-2 (10 U/mL) in 200 μL per well of a 96 well plate. For assays in which T cells were incubated with tumor cell lines, 2-5x10^5 T cells were incubated with tumor cell lines and IL-2 (10 U/mL) with or without peptide (2.5 μg/mL) at T cell to tumor cell ratios of 1:1 – 1:3. After 1 hour of incubation at 37°C, golgi-plug (BD Pharmingen) was added to each well, the plate gently tapped to mix, and incubated for an addition 5-6 hours at 37°C. Cells were incubated with mAb to surface markers and αFcγIII/IIR, fixed and permeabilized (cytofix/cytoperm, BD Pharmingen), washed (perm/wash, BD Pharmingen), incubated with mAb to IFN-γ, and analyzed on a flow cytometer.

Analysis of WT1 expression: RNA was prepared from tumor cells using the Qiagen RNeasy kit (Valencia, CA) and cDNA synthesized using Superscript III (Invitrogen). WT1 message was amplifed using the following primers: ATG CAC TCC TTC ATC AAA CAG GA (205f), TCA GGT CAT GCA TTC AAG CTG (711r), GTG TCT TTT GAG CTG GTC TG (1119r) at an annealing temperature of 51°C. β-actin message

was amplified using GGA AAT CGT GCG TGA CA (forward) and CGT ACT CCT GCT TGC TGA (reverse) at an annealing temperature of 52°C.

Results:

WT1 expression in murine tumors and thymus

WT1 expression has been described in a variety of B6-derived murine tumors including the erythroleukemia FBL (73), the fibrosarcoma BLKSV40 (74), and the prostate tumor cell line TRAMP-C (74). To assess comparative expression in our tumor samples, we isolated RNA from the tumors, synthesized cDNA, and PCR amplified a portion of WT1 using gene specific primers. To compare relative expression levels, samples run for 37, 40, and 43 cycles were analyzed by agarose gel electrophoresis. Equivalent amounts of starting cDNA were amplified for β-actin message.

Among the tumor samples analyzed, BLKSV40 expressed the greatest amount of WT1 message with a faint band visible after 37 cycles of PCR (Figure 6). After 40 cycles, WT1 PCR products were visible for TRAMP-C1A (a tumor line derived from TRAMP-C), and fresh E10 and FBL tumor ascites. After 43 cycles, a PCR product was visible from FBL maintained as ascites but that had been *in vitro* cultured for 5 days, and a weak band was visible for FBL that had been cultured *in vitro* for several weeks. WT1 PCR products were not detected for BLKCL4 (an untransformed murine fibroblast line) and *in vitro* cultured E10 (derived from the previously reported WT1-negative EL-4 thymoma line (73)). The differences observed in WT1 expression for

fresh tumor ascites compared to the equivalent lines cultured *in vitro* may reflect differences in the selective pressures encountered by the tumor cells in these two environments and suggests that assaying for T cell recognition of tumors *in vivo* may be more relevant for designing T cell therapy than only considering data on *in vitro* target cell lysis.

Previous reports also suggest that low levels of WT1 are expressed in murine thymus and/or the capsule surrounding the thymus (106, 107). Thymic expression of WT1 may contribute to central tolerance and the deletion of T cells with a high avidity for this antigen, potentially explaining why such T cells are difficult to isolate. We compared WT1 message levels in murine thymus to those in our tumor samples and detected a PCR product after only 37 amplification cycles (Figure 6). Thus, the levels of WT1 message in whole murine thymus is greater than that found in some murine tumors.

Isolation and characterization of the WT1-specific T cell clone #33

To design a pre-clinical model of adoptive T cell immunotherapy using WT1-specific TCRs to treat murine leukemia, we sought to generate T cells specific for the previously described, H2-D^b-restricted, WT1-derived peptide RMFPNAPYL (73). As efficient methods for the *in vitro* generation and expansion of antigen-specific $CD8^+$ T cells from naïve T cell precursors do not exist for murine T cells, in contrast to recent successes with cultured human T cells (108), mice were immunized to the RMFPNAPYL epitope to generate a memory T cell response to this antigen. Initially,

four mice were immunized with RMFPNAPYL peptide emulsified in incomplete Freund's adjuvant (IFA). RMFPNAPYL-specific T cells were detected in a T cell line from one of these mice, and T cell clones were isolated by limiting dilution. The avidity of the derived clones for antigen was compared by assessing target cell lysis in a chromium release assay using the TAP-deficient cell line RMAS pulsed with ten-fold dilutions of RMFPNAPYL peptide (Figure 7). Most clones killed RMAS cells pulsed with 100 ng/mL RMFPNAPYL peptide but could not lyse RMAS cells pulsed with 10 ng/mL RMFPNAPYL peptide. Clone #33 was chosen for further functional analysis and TCR gene cloning based upon its higher avidity with respect to several other T cell clones in the same line, its ability to proliferate well *in vitro*, and the availability of commercial antibodies to the TCR α and β chains expressed by this clone (Figure 8). This CTL clone produced IFN-γ in response to target cells pulsed with the RMFPNAPYL peptide, but not to cells pulsed with an irrelevant peptide (Figure 9). However, the avidity of this clone was insufficient to lyse the WT1-expressing murine tumor cell lines FBL or BLKSV40 in the absence of exogenous peptide (Figure 10).

To use the clone #33-derived TCR chains as a template for PCR mutagenesis, yeast display, and selection of higher affinity RMFPNAPYL-specific TCRs, the Vα3 and Vβ11 TCR chains were amplified using TCRα and β chain constant region specific primers and RACE PCR. To verify that functional, WT1-specific TCRs had been isolated, the Vα3 and Vβ11 TCR chains were then cloned into separate retroviral vectors, transduced into T cells from P14 TCR transgenic mice, and the transduced T

cells assessed for antigen recognition by intracellular staining for IFN-γ production following peptide stimulation. P14 T cells transduced with both the Vα3 and Vβ11 TCR chains produced IFN-γ in response to stimulation with either the WT1-derived RMFPNAPYL peptide or the LCMV gp33-derived peptide KAVYNFTM (recognized by the endogenous P14 TCR) while mock transduced T cells or T cells transduced with only one of the WT1-specific TCR chains could only recognize the gp33-derived peptide (Figure 11). These data demonstrate that functional chains from a WT1-specific TCR were isolated.

As yeast display relies upon TCR binding to fluorescently-labeled peptide-MHC multimers for affinity selection (88), we asked if the clone #33-derived TCRs bind to RMFPNAPYL-H2-D^b tetramers. P14 T cells were transduced with the Vα3 and Vβ11 TCR chains and the entire T cell population, or T cells sorted for high levels of Vα3 and Vβ11 expression, were incubated with PE-labeled RMFPNAPYL-H-$2D^b$ tetramers at either 4^o or room temperature (Figure 12). No significant tetramer binding was observed, suggesting that the clone #33-derived TCR may be a suboptimal target for mutagenesis and selection in the yeast display system.

Immunizing with activated APCs yields a higher avidity T cell response to RMFPNAPYL

Data from our collaborators in the Kranz lab suggests that the higher the initial affinity of the TCR used for mutation and selection, the higher the resulting affinity one can achieve. Since immunization with IFA and RMFPNAPYL peptide generated

only a low avidity WT1-specific T cell response, and a detectable response in only one of four mice, additional immunization strategies presenting the RMFPNAPYL peptide in a more inflammatory milieu were evaluated to attempt to elicit higher avidity WT1-specific T cells capable of binding to RMFPNAPYL-H-2D^b tetramers, which would be more suitable templates for TCR mutation and selection. These new strategies yielded a number of T cell lines and clones specific for the RMFPNAPYL peptide that were further characterized to determine the highest avidity response elicited and if such T cells could recognize WT1-expressing tumors. In addition, the TCR repertoire of these T cell lines and clones was analyzed using antibodies to TCR Vα and Vβ domains to facilitate TCR gene cloning and to determine if T cells with similar avidities isolated from different, genetically identical mice represented similar populations of T cells recruited in response to WT1-RMFPNAPYL (reproducible TCR gene rearrangements on responding T cells have been noted for several antigens (109-112)) or unique populations resulting from individual differences in TCR repertoire selection.

One strategy employed was immunizing mice with adherent splenocytes (to enrich for antigen presenting cells) that had been activated *in vitro* for 24-36 hours with LPS (10 μg/mL) and αCD40 antibody (5 μg/mL), pulsed with RMFPNAPYL peptide (6 μg/mL) for one hour, and irradiated. Mice were boosted two months later with whole splenocytes that had been similarly treated. One month following the second immunization, splenocytes were stimulated *in vitro* with RMFPNAPYL peptide. The derived M2 line originated from a mouse that received this

immunization regimen, and six days following the first *in vitro* stimulation 5% of the $CD8^+$ T cells in the culture were RMFPNAPYL-specific as demonstrated by intracellular staining for IFN-γ production (Figure 13). After two months of *in vitro* culture and expansion, the M2 line was assayed for avidity in a chromium release assay using RMAS target cells pulsed with varying concentrations of RMFPNAPYL peptide. The lowest concentration of RMFPNAPYL peptide recognized by the M2 line was 1 ng/mL, two logs lower than the concentration recognized by clone #33 (Figures 7 and 14). Antibody staining for TCR Vα and Vβ chains revealed at least three different T cell populations within the M2 T cell line (Vβ5, Vβ8, Vβ13; Figure 15). To determine which of these populations specifically recognized RMFPNAPYL, the M2 line was stimulated with E10 cells pulsed with or without RMFPNAPYL peptide for six hours in the presence of brefeldin A and stained with antibodies to IFN-γ and the identified TCR Vβ domains. Each of the three T cell populations produced IFN-γ in response to RMFPNAPYL peptide stimulation (Figure 16).

To compare the avidities of these three populations with each other, the M2 T cell line was sorted into $V\beta5^+$, $V\beta8^+$, and $V\beta13^+$ sublines. The three different TCR Vβ sublines were stained with antibodies to CD8 and the respective TCR Vβ domains or RMFPNAPYL-H2-Db tetramers (Figure 17). Compared to a P14 T cell line used as a negative control for tetramer binding, the $V\beta5^+$ population was tetramer positive, with an approximately one log shift in staining intensity. The $V\beta8^+$ population was tetramer intermediate, displaying a half log shift, and the $V\beta13^+$ population bound tetramer weakly, exhibiting less than a half log shift. Despite these differences in

tetramer staining, the Vβ5$^+$ and Vβ13$^+$ T cell lines exhibited similar avidities for peptide-pulsed RMAS cells in a chromium release assay (Figure 18). While tetramer staining generally correlates with T cell avidity (32) exceptions have been noted (113), as differences in the expression of molecules in addition to the TCR can contribute to target avidity. Such differences could explain the discrepancy observed between the tetramer staining data and the peptide titration results for the M2 sublines.

Since the M2 line demonstrated a two log higher avidity for antigen compared to clone #33, we asked if this line could lyse WT1 expressing murine tumors. BLKSV40, Tramp-C1A, FBL ascites, and *in vitro* cultured FBL were chromium-labeled and incubated with the M2 line or a P14 T cell line as a negative control in the presence or absence of exogenous RMFPNAPYL peptide. No tumor cell lysis was observed in the absence of exogenous peptide, demonstrating that the M2 T cell line still lacked sufficient avidity to lyse murine tumors endogenously expressing WT1 (Figure 19). Similarly, the three M2-derived sublines and a tetramer positive, Vβ5$^+$ T cell clone (clone 4) derived from the M2 T cell line failed to secrete IFN-γ in response to the endogenous levels of WT1 expression in fresh FBL ascites (Figure 20), but did produce IFN-γ if exogenous RMFPNAPYL peptide was added to the targets.

Testing a recombinant Listeria as a tumor vaccine

Since immunization with activated APCs yielded a higher avidity T cell response to RMFPNAPYL than peptide in IFA, we examined if RMFPNAPYL peptide presented to T cells by activated APC within the context of an ongoing

infection would yield an even higher avidity T cell response to this antigen. To generate a potentially useful vaccine for eliciting RMFPNAPYL-specific $CD8^+$ T cells, a recombinant *Listeria monocytogenes* bacterium was created with the RMFPNAPYL epitope embedded within a segment of the ovalbumin gene engineered into the $tRNA^{Arg}$ locus of the *Listeria* genome (Cerus Corp.). Mice were primed with activated and peptide pulsed APC as previously described for the M2 cell line and boosted one month later with recombinant *Listeria*. On day 6 following infection, splenocytes from infected mice were stimulated *in vitro* with RMFPNAPYL peptide, SIINFEKL peptide (a MHC class-I binding epitope present within the ovalbumin protein), or no peptide. Roughly 5-6% of the $CD8^+$ T cells produced IFN-γ in response to SIINFEKL stimulation, but no response was seen to RMFPNAPYL stimulation (Figure 21a). Priming and boosting mice with this recombinant vector also failed to stimulate RMFPNAPYL-specific T cell responses, but generated a robust response to SIINFEKL (Figure 21b). Similar responses were seen in mice infected with a Listeria-ovalbumin control bacterium (data not shown).

Studies by collaborators at Cerus determined that better expression could be achieved with a different recombinant construct, in which the target epitope is positioned further from SIINFEKL and driven by a different promoter (see Materials and Methods). Therefore, a second generation recombinant *Listeria* expressing the RMFPNAPYL epitope was tested. Three mice were immunized twice with activated splenocytes and boosted a month after the second immunization with this second generation recombinant *Listeria*. Two previously naïve mice were also infected with

the recombinant *Listeria*. On day six following infection, splenocytes were restimulated *in vitro* with RMFPNAPYL, SIINFEKL, or KAVYNFTM (gp33-derived peptide as a negative control) peptides and assessed for IFN-γ production by intracellular staining (Figure 22). Two of three mice that had been primed to RMFPNAPYL before boosting with the Listeria exhibited RMFPNAPYL-specific T cell responses (5.4 and 1.3% of the $CD8^+$ T cells), and one of two mice immunized only with the recombinant *Listeria* responded to RMFPNAPYL (1.5% of the $CD8^+$ T cells). Further experiments examining the ability of this vector to generate high avidity WT1-specific T cells are ongoing.

Characterization of additional RMFPNAPYL-specific T cell lines

Lines 1A, 2A, 3C, and 3D originated from 3 mice initially primed with activated, peptide-pulsed APC as described for the generation of the M2 T cell line. These mice were subsequently boosted with the first generation recombinant *Listeria* described above. While RMFPNAPYL-specific T cell responses were not detected in mice 1 and 2 on day six following immunization (Figure 21), *in vitro* culture and stimulation of splenocytes from these mice with RMFPNAPYL peptide resulted in the outgrowth of WT1-specific T cells as demonstrated by lysis of RMFPNAPYL peptide-pulsed targets (data not shown). Like the M2 line, both the 1A and 2A lines consisted of several different T cell populations as assessed by TCR Vα and Vβ chain expression (Figure 23). The 1A line consisted predominantly of equivalent numbers of $V\beta5^+$ and $V\beta8^+$ T cells as well as a small population of $V\alpha3^-V\beta11^+$ T cells (5%).

In contrast, the 2A line consisted predominantly of $V\beta8^+$ T cells (67%) with smaller numbers of $V\beta13^+$ (8%) and $V\alpha3^+V\beta11^-$ (4%) T cells. When analyzed for the ability to bind RMFPNAPYL-H2-D^b tetramers, 43% of cells within the 1A line were positive while no cells within the 2A line bound tetramer (Figure 23). To identify the population(s) of T cells within these lines that recognized RMFPNAPYL, both lines were assayed for IFN-γ secretion in response to stimulation with *in vitro* cultured E10 cells or FBL ascites pulsed with or without WT1 peptide (Figure 24). Both the $V\beta5^+$ and $V\beta8^+$ T cells within the 1A line produced IFN-γ in response to RMFPNAPYL peptide stimulation but not in response to endogenous WT1 expression by FBL grown in ascites. All cells within the 2A T cell line failed to produce IFN-γ, even in response to peptide-pulsed targets, and the line expanded poorly *in vitro*, suggesting that it consisted predominantly of T cells with a low avidity for the RMFPNAPYL epitope. As we were primarily interested in T cells with a high avidity for this antigen, further analyses of the 2A line were not performed.

To further characterize the T cell populations within the 1A line and to identify the tetramer positive subset, seven T cell clones were isolated by limiting dilution and avidities for RMFPNAPYL compared by examining lysis of RMAS cells pulsed with varying concentrations of RMFPNAPYL peptide (Figure 25 and data not shown). The 1A-derived T cell clones exhibited a wide range of avidities, spanning approximately a three log difference in peptide concentration. The lowest avidity T cell clone (1A-9C) expressed Vβ11, required a minimum of 100 ng/mL RMFPNAPYL peptide for target cell lysis, and failed to bind tetramer (data not shown). Clones 1A-5E and 1A-8E

exhibited intermediate avidities, requiring 1 ng/mL and 10 ng/mL RMFPNAPYL peptide, respectively (Figure 25), expressed a Vβ5 TCR chain and also failed to bind tetramer (Figure 26 and data not shown). The highest avidity clones isolated (1A-1E, 1A-4E, 1A-8C, and 1A-10H) recognized targets pulsed with as little as 100 pg/mL of peptide (Figure 25). All four clones expressed Vβ8 and bound tetramer (Figure 26 and data not shown). Additional analysis to determine more precisely the TCR chains used by these clones and if they are progeny of a common precursor is ongoing. The four highest avidity clones were tested for the ability to lyse BLKSV40. Despite binding tetramer and exhibiting a log higher avidity than the M2 line, these clones were still unable to lyse a WT1-expressing murine tumor (Figure 27).

In each of the 3 mice from which the M2, 1A, and 2A T cell lines originated, several different TCRs were used to respond to the RMFPNAPYL peptide. A $V\beta8^+$ TCR was used by all 3 mice, however, it was not always associated with a high avidity response capable of binding tetramer. A $V\beta5^+$ TCR and a $V\beta13^+$ TCR were each detected in two mice. To determine if these TCR chains are reproducibly found in the murine T cell response to WT1-RMFPNAPYL, T cell lines from two additional immunized mice were examined. Line #5770C originated from a mouse immunized twice with activated, peptide-pulsed whole splenocytes (not enriched for APC) and boosted with RMFPNAPYL peptide in IFA. Staining with antibodies to various TCR Vα and Vβ domains revealed that it consisted of predominantly two different populations, a $V\beta8^+$ population and a $V\alpha11^+V\beta7^+$ population (Figure 28 and data not shown). Lines 3C and 3D originated from a mouse primed with activated, peptide-

pulsed APC and boosted with the first generation *Listeria* vector. Antibody staining to TCR Vα and Vβ domains revealed that line 3C consisted entirely of a $V\beta7^+$ (and $V\alpha11^-$) T cell population, while the TCR usage of line 3D could not be determined based on available anti-TCR antibodies (Figure 29 and data not shown).

As the TCRs expressed by lines 3C and 3D were not detected in the previous T cell lines analyzed, we examined if these lines represented higher or lower avidity T cells compared to the other T cell lines. The avidities of lines 3C and 3D were compared to the $V\beta8^+$ high affinity clones from the 1A line and the $V\beta5^+$ clone 4 from the M2 line using RMAS target cells pulsed with varying concentrations of RMFPNAPYL peptide (Figure 29). Line 3C required a minimum of 1 ng/mL of RMFPNAPYL peptide for target cell lysis, while line 3D lysed targets pulsed with as little as 100 pg/mL RMFPNAPYL peptide.

To determine if tetramer staining intensity correlated with T cell avidity for the higher avidity T cell lines and clones, lines #5770C, 3C, and 3D and T cell clones 1A-8C, 1A-10H, and M2 clone 4 were incubated with RMFPNAPYL-H2-D^b tetramers and the mean fluorescence intensity (MFI) of the tetramer signal measured (Figure 30). T cell lines from $TCR_{\alpha gag}$ and P14 TCR transgenic mice were used as negative controls. In general, higher avidity T cells stained more brightly with tetramer than lower avidity T cells, however, this correlation was not absolute. For example, the highest avidity T cells (1A-8C, 1A-10H, and 3D) yielded the highest intensity fluorescence (226, 169, and 369, respectively), but the MFI of clone 1A-10H (169) was similar to that of M2 clone 4 (160) which exhibited a log lower avidity by peptide

titration. Line #5770C consisted of two different populations, one that bound tetramer strongly and exhibited a MFI similar to that of the highest avidity clones (299), and another population that weakly bound tetramer (MFI = 22, roughly 7 fold higher than background levels).

The peptide titration, tetramer staining, and TCR Vβ staining data for the T cell lines and clones analyzed is summarized in Table 1. It reveals that the murine response to WT1-RMFPNAPYL varies considerably between mice and that the T cell response within a given mouse is often polyclonal, consisting of T cells with a range of avidities and employing many different TCR chains.

Discussion:

Despite using a variety of different immunization strategies, and analyzing T cell responses from multiple mice, we were unable to elicit T cells capable of recognizing murine tumors endogenously expressing WT1. Gaiger and colleagues (74) reported similar results, eliciting T cells with peptide immunization that recognized RMFPNAPYL peptide-pulsed target cells but not tumor cells and failed to protect mice from tumor challenge. Two studies from Sugiyama's laboratory, which demonstrated either protection from tumor challenge (75) or successful tumor therapy (76), utilized tumors transfected with WT1 cDNA, likely expressing higher levels of WT1 protein than what is typically found in tumors endogenously expressing this antigen. T cells with an avidity sufficient to recognize such transfected tumors may still be unable to recognize tumors endogenously expressing low levels of WT1.

There is only a single published report demonstrating the ability to generate $CD8^+$ T cells from the endogenous murine T cell repertoire with the capacity to recognize murine tumors endogenously expressing WT1 (73). Nonetheless, the FBL tumor prevention study in this report is problematic. The authors claim that mice immunized with the RMFPNAPYL peptide pulsed onto LPS-activated splenocytes protect mice from a large ($3x10^7$ cells) FBL tumor challenge. However, the published survival curves only follow the cohort of immunized mice out to 14 days post tumor injection (5 days after the death of the last control immunized mouse). It is unclear from the text what percentage of immunized mice survived long-term following tumor challenge. It is also unclear from the data they present whether their ability to isolate a T cell line capable of lysing FBL *in vitro* was a fortuitous event or if the levels of WT1 expression in their FBL tumor line are higher than those in ours. Unfortunately, their FBL line is no longer available for comparison.

Like most tumor associated antigens, WT1 is a self protein that is normally expressed in some peripheral tissues (8). Many self proteins, despite appearing to be tissue-specific and/or lineage-restricted, are also expressed in the thymus, which can result in central tolerance (114). Two published reports have examined WT1 expression in the mouse thymus. One group demonstrated very low expression of WT1 RNA by Northern blot compared to the amounts present in kidney and spleen (106). Another group used *in situ* hybridization to examine WT1 mRNA expression and reported that WT1 expression in the thymus was restricted to the thymic capsule and was not present within the thymus proper, although high 'background' levels of

autofluoresence were observed within the thymus (107). Preliminary data from our lab suggests that WT1 is also expressed in developing thymocytes and ongoing work is examining the particular thymic subsets in which it is expressed. Although peripheral expression of WT1 in normal tissues has been described as being at 10-1000 fold lower levels than what is typically found in $WT1^{+}$ tumors (9, 74), we found message levels for WT1 in the thymus greater than what was present in most tumor samples we analyzed. These relatively high levels of WT1 expression within the thymus likely contribute to T cell tolerance to this protein, causing deletion of the very high avidity repertoire, including most of the T cells that could potentially recognize WT1-expressing tumors.

In spite of these levels of WT1 expression within the thymus, T cells elicited from multiple mice were capable of recognizing the RMFPNAPYL epitope. However, a wide range of functional avidities were observed for the RMFPNAPYL peptide, spanning three orders of magnitude in some cases. In general, lower avidity T cells expanded poorly *in vitro* and could not be maintained in culture for more than a few months. Only higher avidity T cell lines and clones proliferated well *in vitro* and bound RMFPNAPYL-H-$2D^{b}$ tetramers. Since tetramer binding facilitates selection of high affinity TCR mutants in the yeast display system, and the starting affinity may affect the final affinity or "affinity ceiling" one can reach, the TCRs from the higher avidity T cells will likely make better templates for mutation, yeast display, and affinity selection than the clone #33 TCR, or the TCRs from the other low affinity T cell clones.

The T cell response to some antigens is dominated by a single TCR clonotype that is reproducibly found in multiple mice (109-112), but such relatively restricted responses and "public" TCRs were not found in the murine response to $WT1_{RMFPNAPYL}$. The described focused T cell responses have generally been directed toward foreign antigens found in pathogens, and may reflect evolutionary pressure to preserve efficient recognition of these antigens, or simply the fact that infection represents the first time the immune system has seen these antigens and has had an opportunity to respond to them. As WT1 is a self antigen expressed in both the thymus and the periphery, tolerance mechanisms presumably have multiple opportunities to shape the available repertoire of RMFPNAPYL-specific T cells. The variety of different TCRs recruited in response to RMFPNAPYL peptide immunization (both within individual mice and among all the mice examined) may reflect the fact that T cells with a very high avidity, that might normally dominate the response were WT1 a foreign antigen, have been eliminated, leaving only lower avidity or "sub-dominate" T cell responses. Recent crystallographic studies of three different autoimmune TCRs bound to the respective peptide-MHC complexes support this notion. The suboptimal recognition of bound peptide and unusual binding topologies exhibited by these TCRs may have allowed them to escape negative selection, while T cells expressing more optimal receptors were deleted (115).

We are currently generating a panel of related, high affinity, RMFPNAPYL-specific TCRs exhibiting differences in TCR on and off rates and thermodynamic interactions with peptide-MHC. These reagents will be useful tools for addressing

several questions relevant to targeting WT1 for cancer immunotherapy, as well as yielding insights about the biology the immune response to this protein. Using this panel, we hope to discern how wide the window of differential recognition of tumor cells and normal tissues expressing WT1 is, and how the thermodynamics of TCR interaction with peptide-MHC affects T cell avidity and recognition of cells expressing low levels of antigen. Furthermore, these tools will allow us to address if tolerance mechanisms targeting the RMFPNAPYL antigen are "overprotective", eliminating from the endogenous repertoire T cells with the capacity to recognize tumors but still ignore normal tissues. TCRs could be transduced into peripheral T cells, as well as murine hematopoietic stem cells (116-118), to ask what happens to T cells expressing TCRs with high affinity for self antigen during different points of T cell development.

In summary, the murine response to the WT1-derived RMFPNAPYL peptide covers a wide range of avidities and recruits T cells expressing a variety of different TCRs. Optimization of vaccine strategies should improve the quality of the response generated, but we are currently unable to isolate $CD8^+$ T cells capable of recognizing the low levels of WT1 expressed in our murine tumors. Thus, further study of adoptive T cell immunotherapy targeting this antigen in the mouse will likely require *in vitro* engineered TCRs with a higher affinity for this antigen. We now have a number of different RMFPNAPYL-specific TCRs, several of which bind to tetramers and may be good templates for yeast display and tetramer-based selection by fluorescence activated cell sorting. If these TCRs fail to bind tetramer when expressed in yeast, another recently described method based on density centrifugation can also

be pursued (119). Our previous extensive experience with adoptive immunotherapy of disseminated FBL leukemia, which endogenously expresses low levels of WT1, suggests that it may be a good model system for testing *in vitro* engineered, WT1-specific TCRs for the ability to selectively lyse tumor cells without targeting normal tissues.

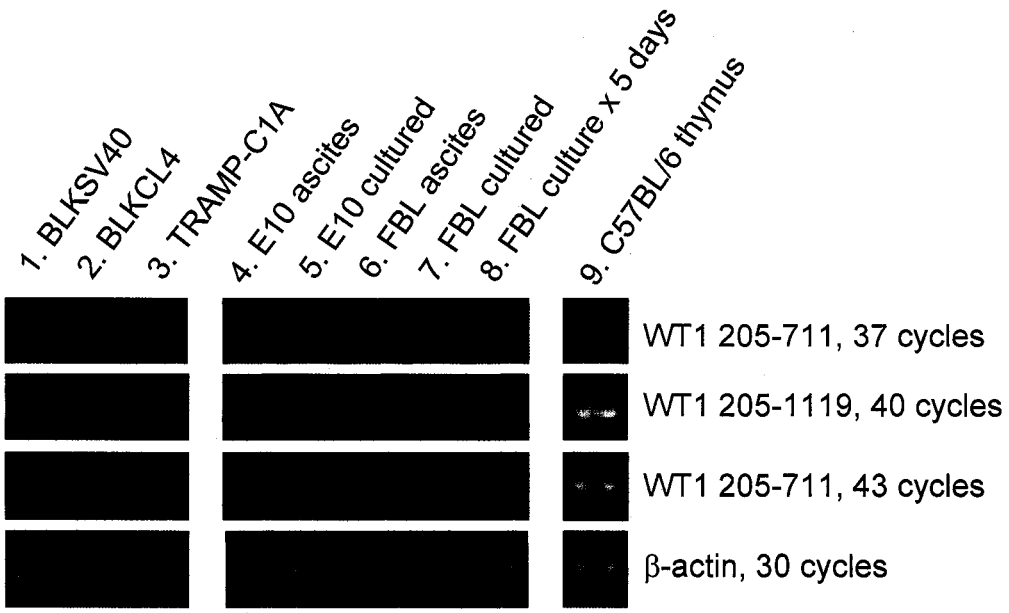

Figure 6: WT1 expression in murine tumors and thymus. Equivalent amounts of RNA were used as starting material to make cDNA and probe for WT1 expression. In this semi-quantitative assay, primers were used to amplify base pairs 205-711 or 205-1119 of the WT1 gene or β-actin as an amplification and loading control. All cells are derived from C57BL/6 mice. Their origin is as follows: 1) BLKSV40 is a SV40-transformed fibrosarcoma line, 2) BLKCL4 is the untransformed control fibroblast cell line for BLKSV40, 3) TRAMP-C1A is a prostate tumor cell line, 4 & 5) E10 is a thymoma line derived from EL4, "ascites" refers to fresh tumor ascites and "cultured" to cells passaged long-term *in vitro*, 6-8) FBL is an erythroleukemia, lane 8 consists of FBL ascites cultured *in vitro* for only 5 days, 9) whole thymus of a 4 week old C57BL/6 mouse.

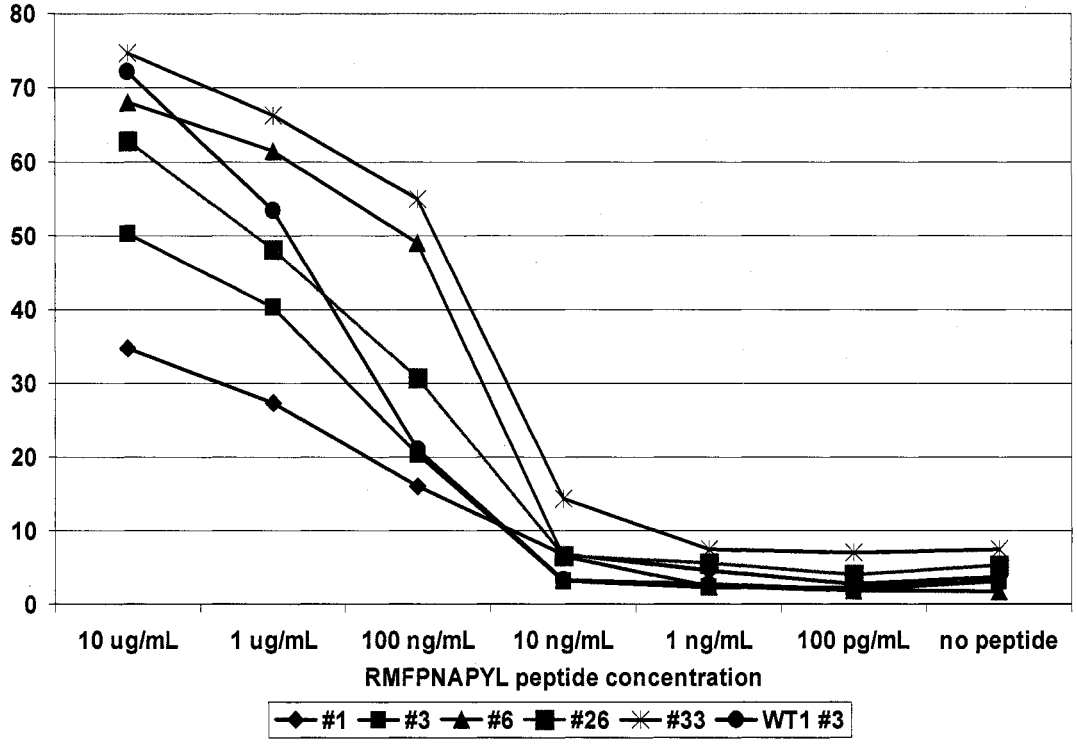

Figure 7: Isolation of the WT1-specific T cell clone #33. RMFPNAPYL-specific T cells were detected in a T cell line from one of four mice immunized with IFA+RMFPNAPYL peptide and cloned by limiting dilution. The avidity of clones #1, 3, 6, 26, and 33 were compared to the parental T cell line (WT1 #3) in a chromium release assay using the murine TAP-deficient cell line RMAS pulsed with varying concentrations of RMFPNAPYL peptide at a T cell to target ratio of 10:1.

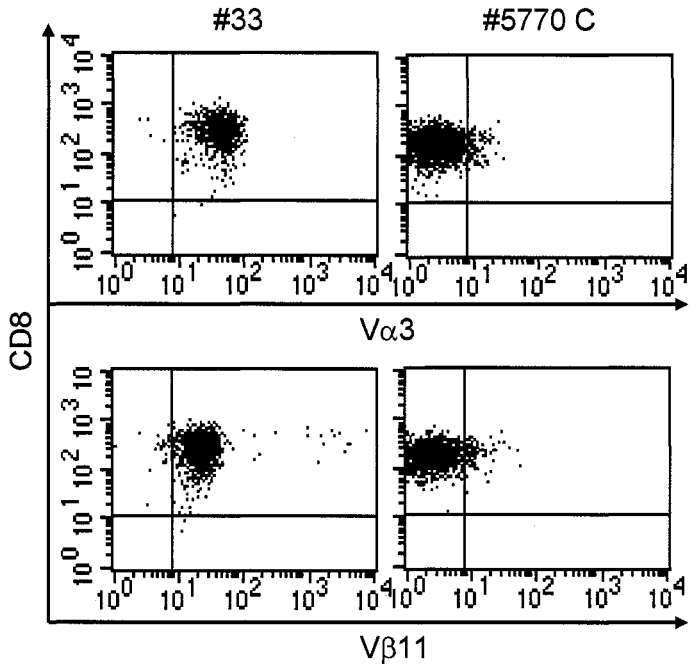

Figure 8: Clone #33 expresses TCR chains Vα3 and Vβ11. Clone #33 was stained with antibodies to CD8α and Vα3 or Vβ11. T cell line #5770C served as a negative control for TCR antibody staining.

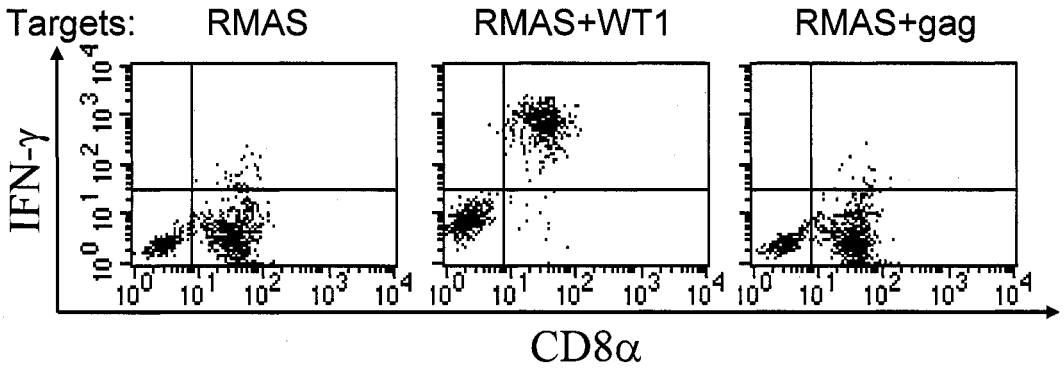

Figure 9: Clone #33 produces IFN-γ upon antigen stimulation. IFN-γ production was assessed by intracellular cytokine staining after stimulating clone #33 for 5 hours with RMAS cells pulsed with no peptide, 10 μg/mL WT1-derived RMFPNAPYL peptide, or 10 μg/mL FMuLV gag-derived peptide (CCLCLTVFL) as a negative control.

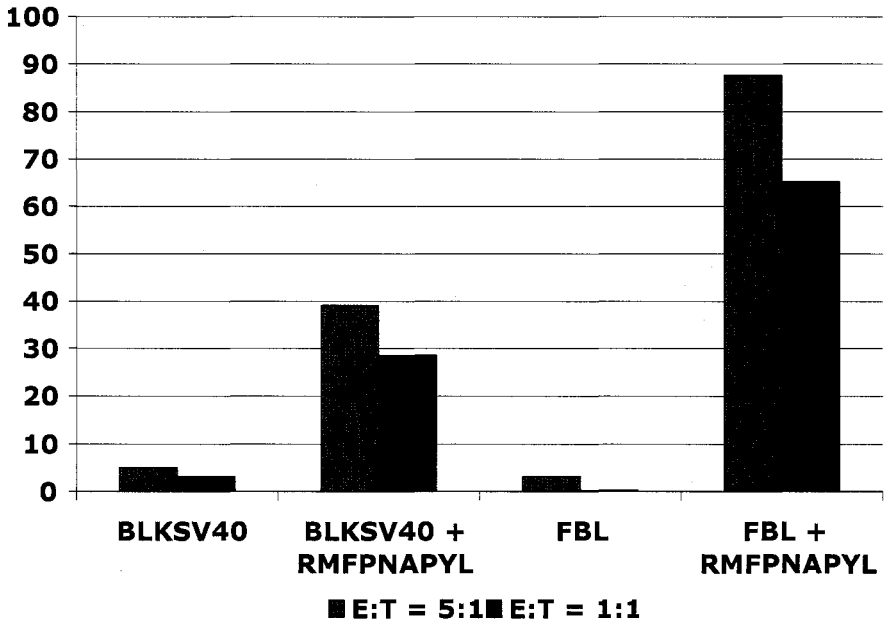

Figure 10: Clone #33 fails to lyse murine tumors endogenously expressing WT1. Clone #33 was tested for the ability to lyse the murine tumors BLKSV40 or FBL (*in vitro* cultured) in a four-hour chromium release assay. T cell (E) to tumor (T) ratios were 5:1 or 1:1. Tumor cells can be lysed if exogenously pulsed with the RMFPNAPYL peptide.

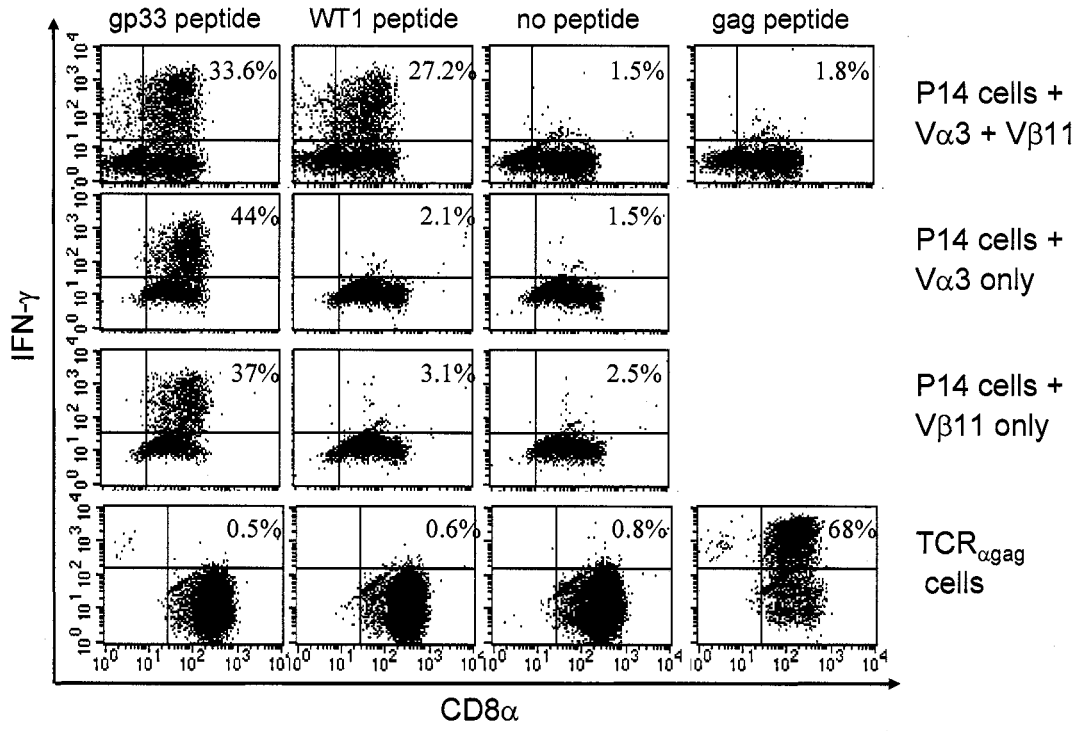

Figure 11: Transfer of the clone #33 TCR chains to P14 T cells confers RMFPNAPYL antigen specificity. Splenocytes from P14 TCR transgenic mice were transduced with separate retroviruses encoding the clone #33-derived TCR chains Vα3 and/or Vβ11, stimulated with WT1 (RMFPNAPYL), gp33 (KAVYNFATM), or gag (CCLCLTVFL) - derived peptides for 6 hours in the presence of brefeldin A and analyzed for intracellular production of IFN-γ. $TCR_{\alpha gag}$ T cells serve as an internal control for the assay.

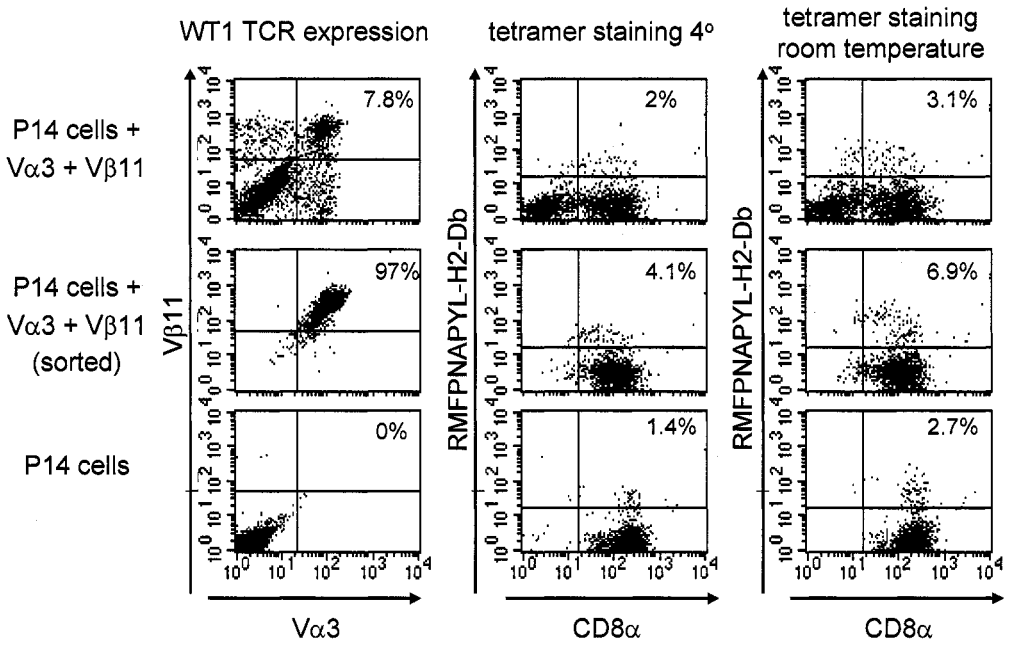

Figure 12: T cells expressing the TCR chains from clone #33 do not bind to RMFPNAPYL-H2-Db tetramers. P14 T cells were mock transduced or transduced with the clone #33 TCR. Some transduced T cells were sorted for high levels of transduced TCR expression and rested *in vitro* to allow re-expression of the transduced TCR chains. T cells were stained with tetramer (1:33 dilution) in 50 μL reactions with an α-CD8α antibody (1:50) for 30 minutes at 4°C or room temperature in the dark.

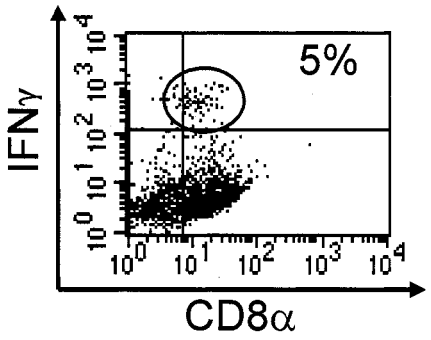

Figure 13: Expansion of RMFPNAPYL-specific $CD8^+$ T cells from an immunized mouse. On day 6 following the first *in vitro* stimulation with RMFPNAPYL peptide, the M2 cell line was stimulated with RMFPNAPYL peptide for 6 hours in the presence of brefeldin A and analyzed for IFN-γ production by intracellular cytokine staining. 5% of the $CD8^+$ T cells were RMFPNAPYL-specific.

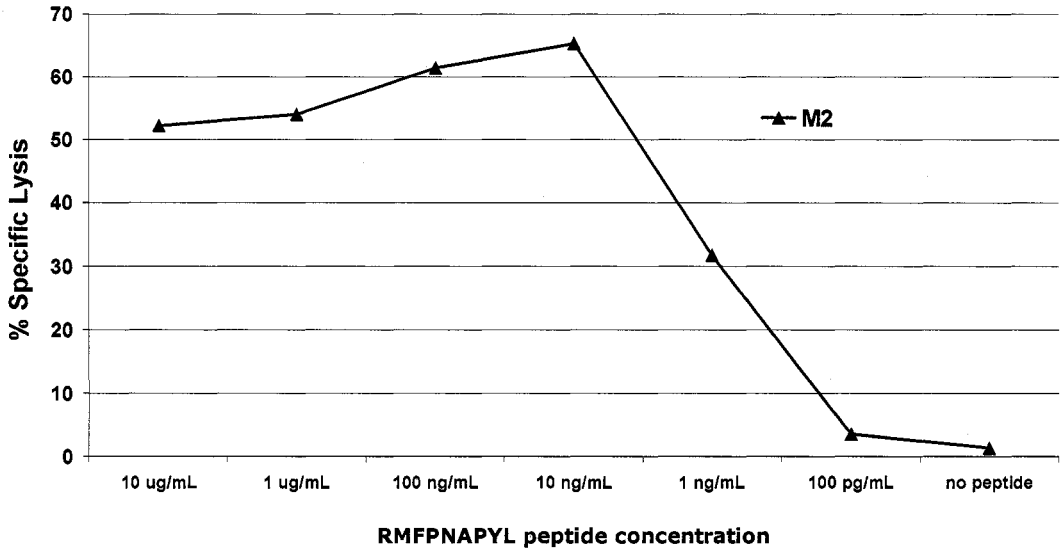

Figure 14: The M2 T cell line has a higher avidity than clone #33. The M2 T cell line was incubated with ^{51}Cr-lableled RMAS cells pulsed with the indicated concentrations of RMFPNAPYL peptide in a four hour chromium release at a T cell to target cell ratio of 10:1.

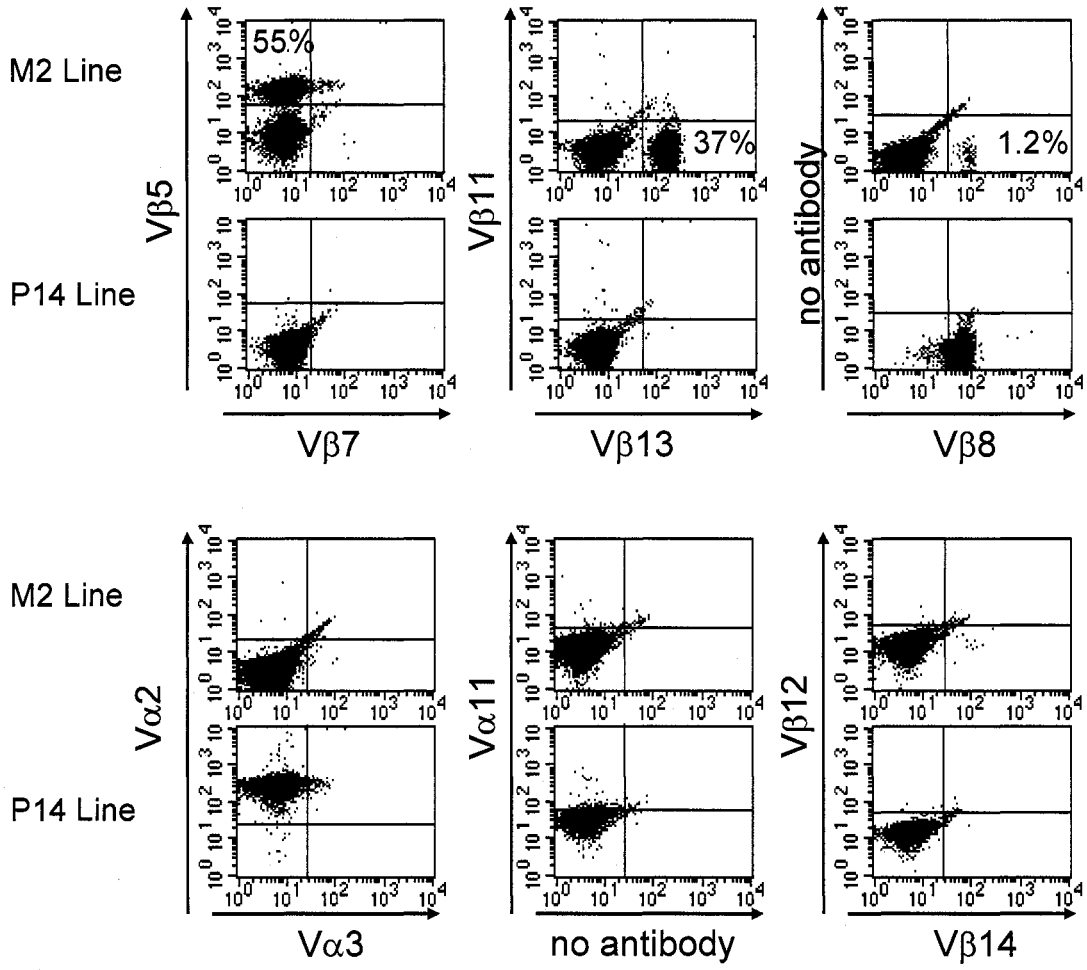

Figure 15: Three distinct T cell populations exist within the M2 T cell line. The M2 T cell line was stained with a panel of anti-TCR Vα and Vβ antibodies (1:100 dilution) at 4°C for 30 minutes. A P14-derived T cell line ($V\alpha2^{+}V\beta8^{+}$) was used as a staining control.

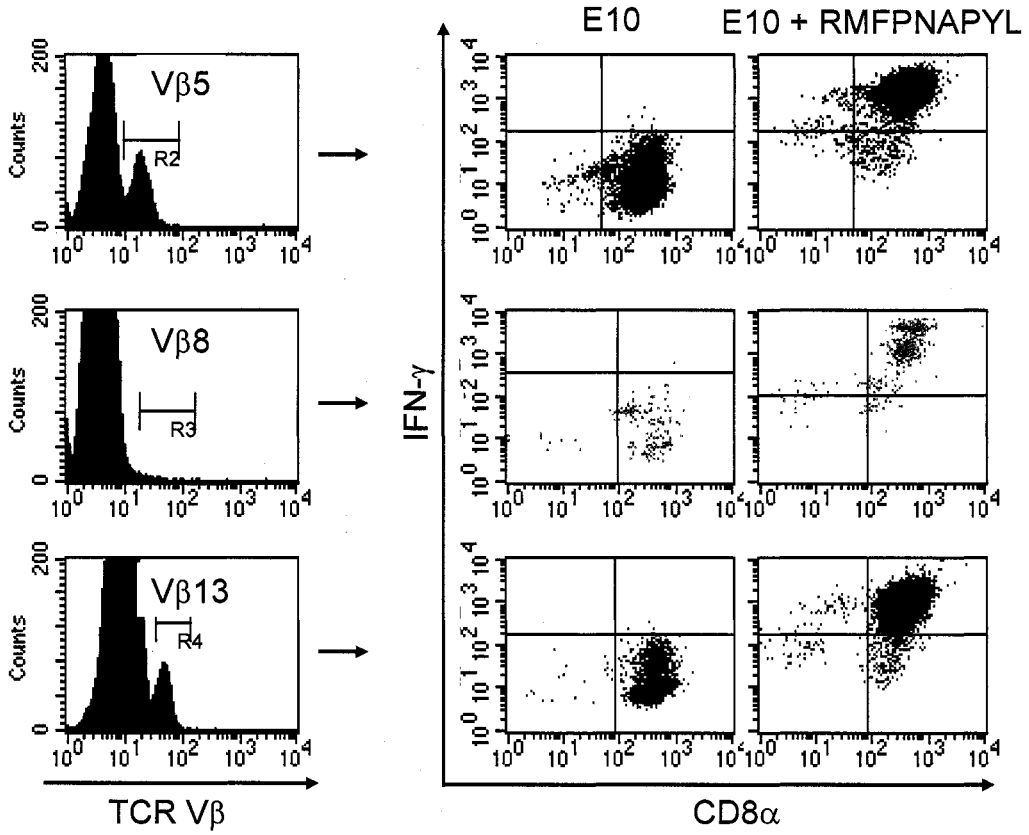

Figure 16: All three T cell populations within the M2 T cell line are RMFPNAPYL-specific. The M2 T cell line was stimulated with *in vitro* cultured E10 tumor cells pulsed with or without RMFPNAPYL peptide for 6 hours in the presence of brefeldin A and assessed for IFN-γ production by intracellular cytokine staining. T cells were also stained with antibodies to CD8α and one of the 3 identified TCR chains (Vβ5, Vβ8, or Vβ13).

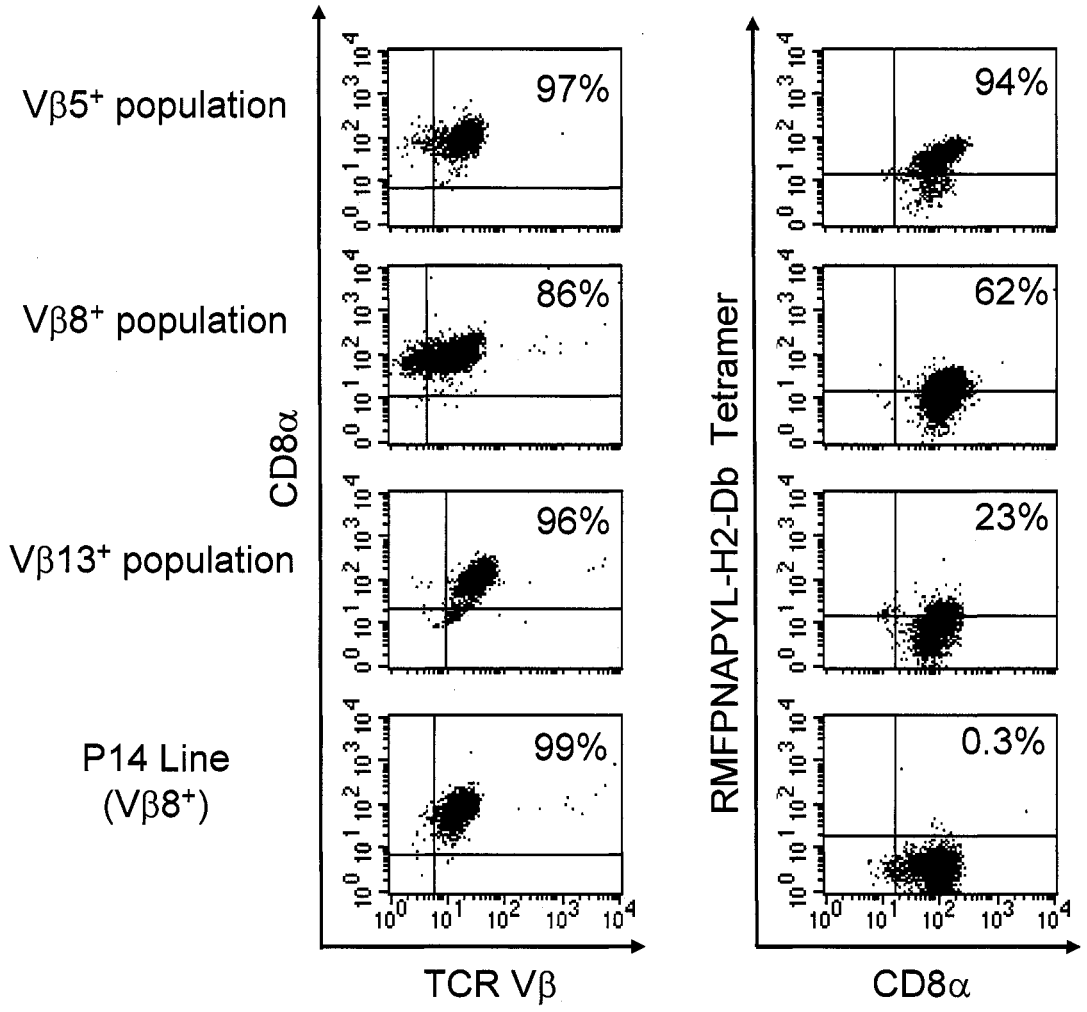

Figure 17: The WT1-specific T cell populations within the M2 T cell line bind RMFPNAPYL-H2-D^b tetramers with different avidities. The M2 line was sorted by flow cytometry to isolate predominantly Vβ8$^+$ and Vβ13$^+$ T cell populations. The M2 line became predominantly Vβ5$^+$ over time in culture. T cell lines were stained with antibodies to CD8α and TCR Vβ5, 8, or 13 or with RMFPNAPYL-H2-D^b tetramers. A P14 T cell line serves as a staining control.

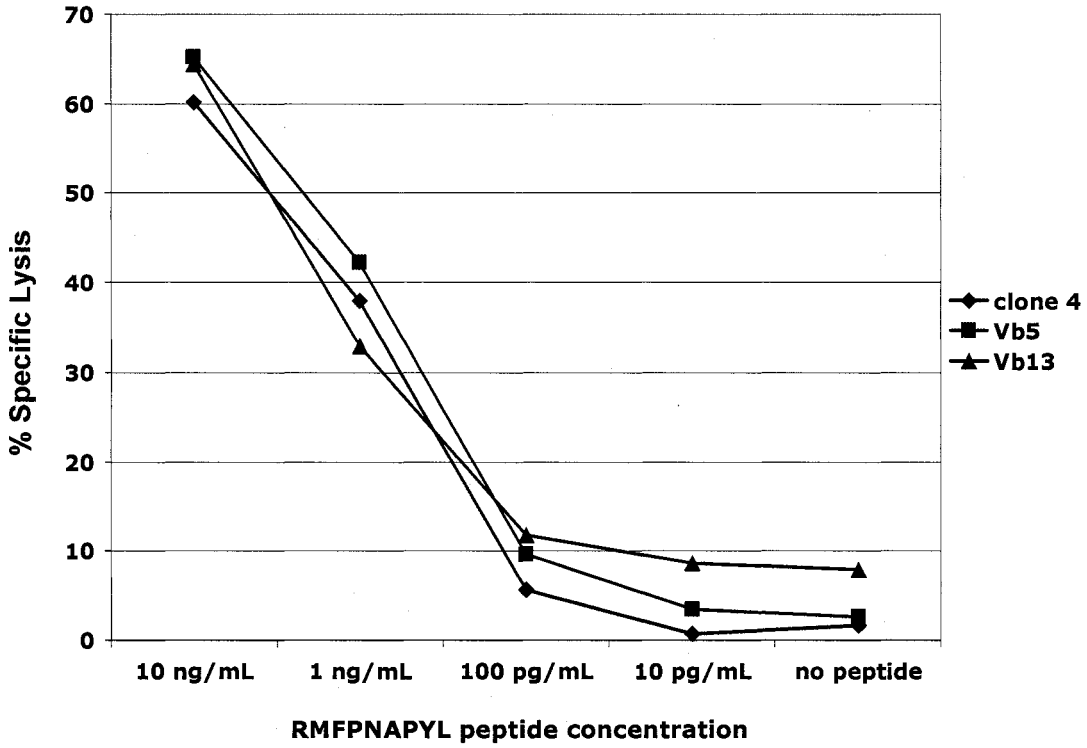

Figure 18: The $V\beta5^+$ and $V\beta13^+$ T cell populations within the M2 line exhibit similar avidities. The avidities of the $V\beta5^+$ and $V\beta13^+$ T cell sublines and a T cell clone derived from the $V\beta5^+$ subline (clone 4) were compared in a 4 hour chromium release assay using RMAS cells pulsed with different concentrations of the RMFPNAPYL peptide at T cell to target cell ratio of 10:1.

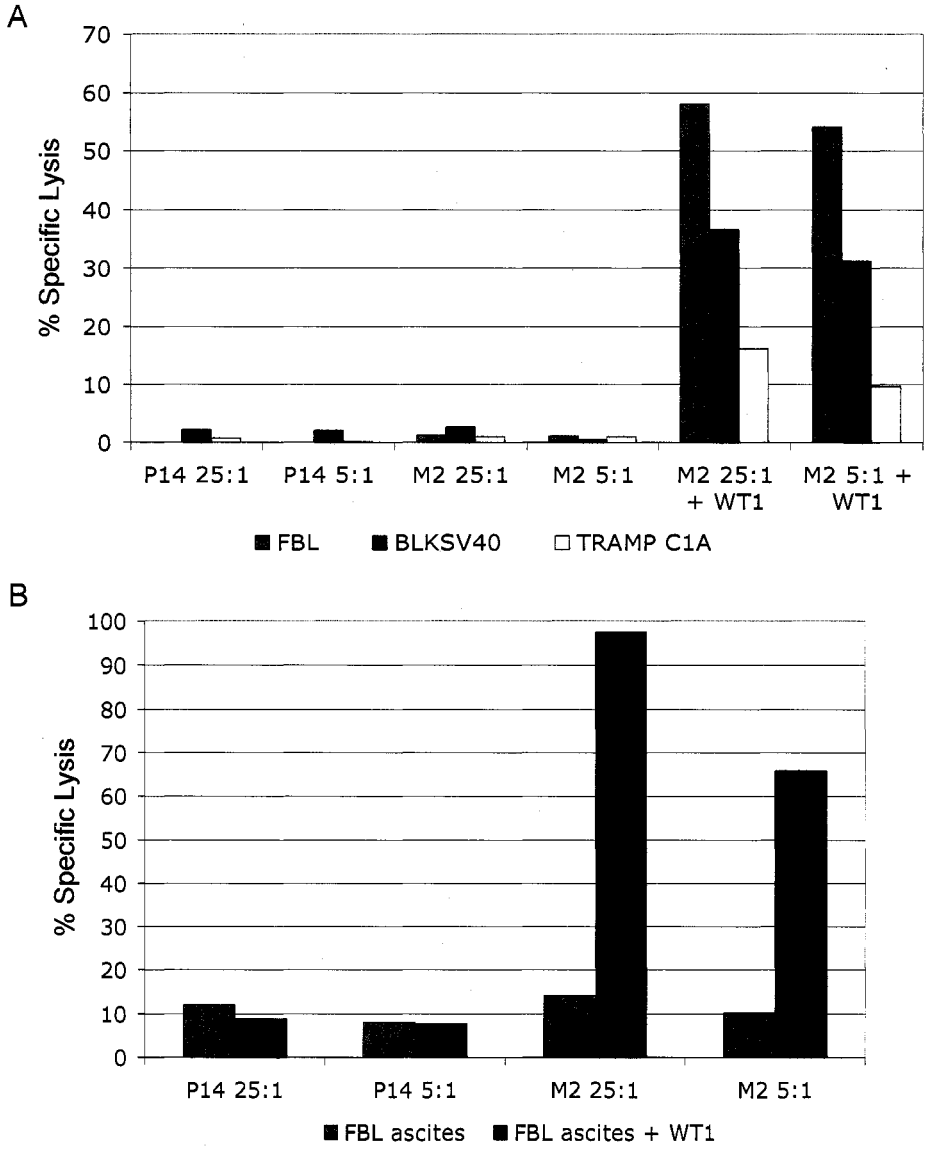

Figure 19: The M2 T cell line does not recognize murine tumors endogenously expressing WT1. A) The M2 T cell line (~70% V$\beta5^+$) was tested for the ability to recognize the WT1-expressing murine tumors FBL (*in vitro* cultured), BLKSV40, or TRAMP-C1A in the presence or absence of exogenous peptide (10 μg/mL) in a 4 hour chromium release assay at T cell to tumor target ratios of 25:1 and 5:1. The P14 T cell line serves as a negative control. B) Same as in A except that the M2 line was ~90% V$\beta5^+$ and only recognition of fresh FBL ascites was examined.

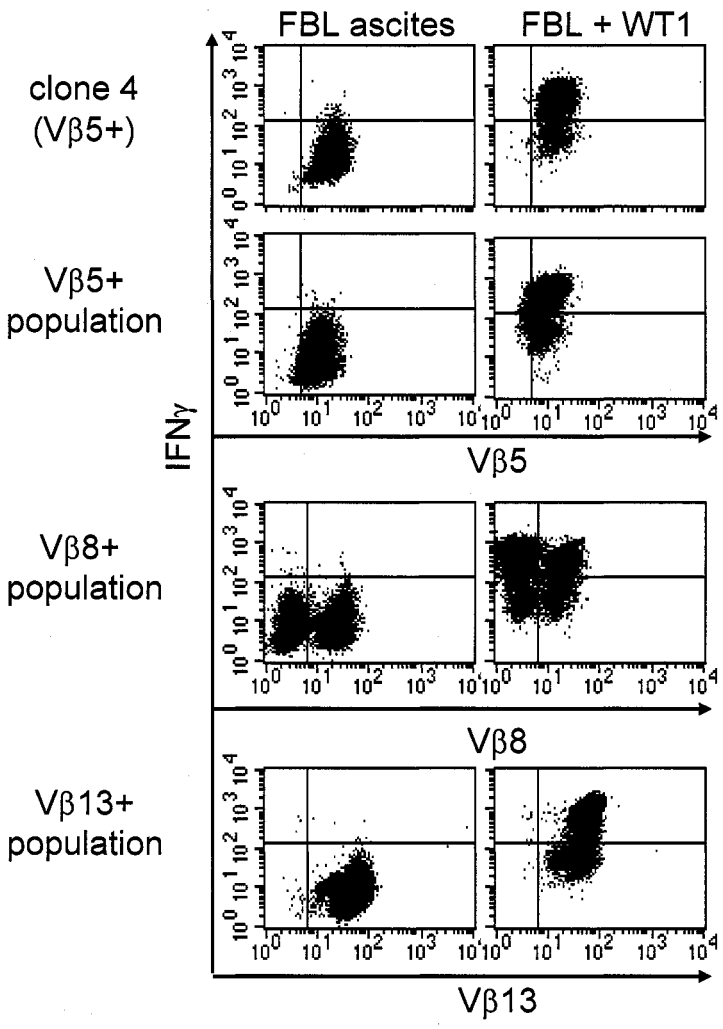

Figure 20: The M2 line does not produce IFN-γ in response to stimulation with FBL ascites. The sorted M2-derived T cell sublines or T cell clone 4 were incubated with fresh FBL ascites with or without exogenous WT1 peptide (1 μg/mL) for 6 hours in the presence of brefeldin A and analyzed for intracellular production of IFN-γ. The sorted $V\beta8^+$ population initially contained a small population of $V\beta5^+$ T cells that outcompeted the $V\beta8^+$ cells over time.

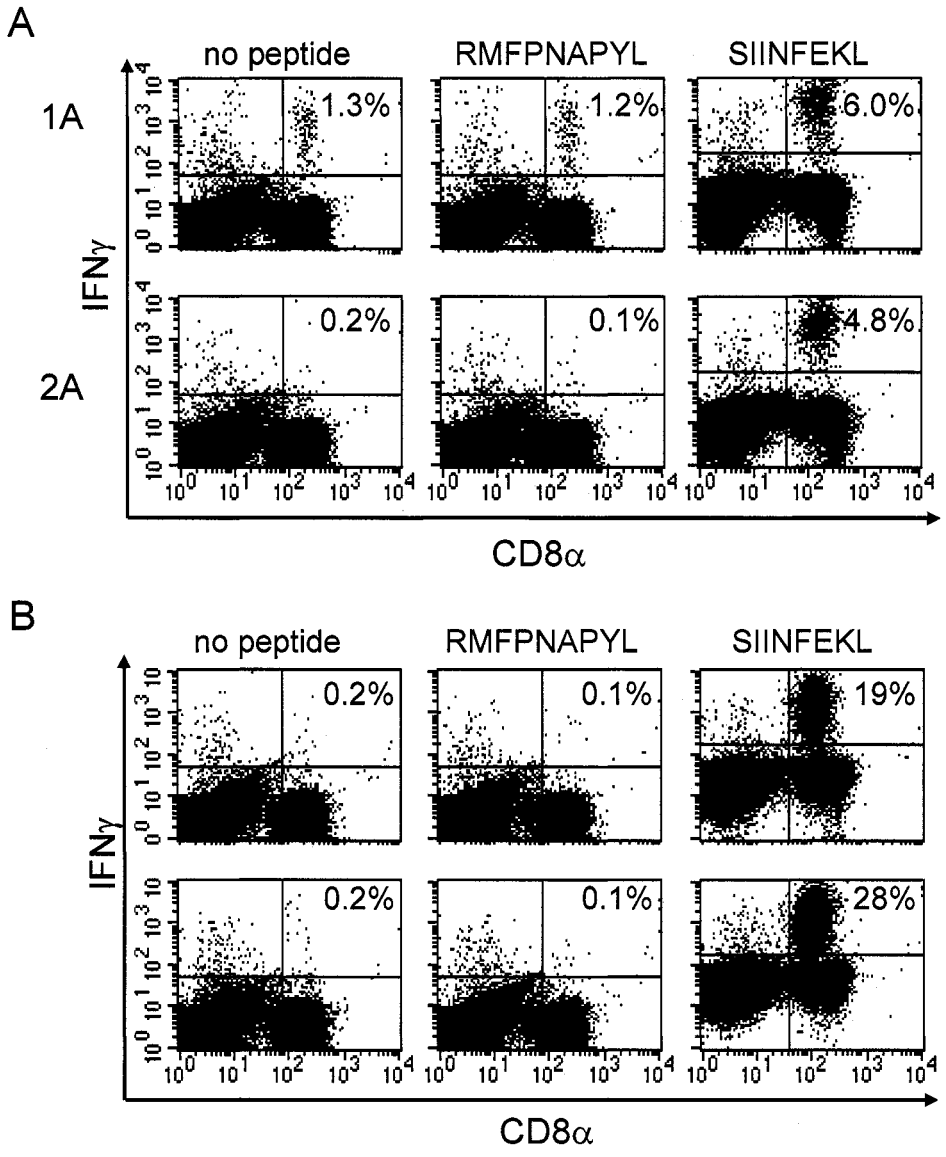

Figure 21: A first generation recombinant WT1-Listeria vector does not stimulate or boost RMFPNAPYL-specific T cell responses. A) Mice were immunized with RMFPNAPYL peptide-pulsed activated adherent splenocytes and infected 4 weeks later with recombinant Listeria. Six days post infection, splenocytes were restimulated *in vitro* with RMFPNAPYL, SIINFEKL, or no peptide for 6 hours in the presence of brefeldin A and analyzed for IFN-γ production. B) Mice were infected with recombinant Listeria, boosted with the same vector and analyzed as in A. Percentages are % of $CD8^+$ T cells.

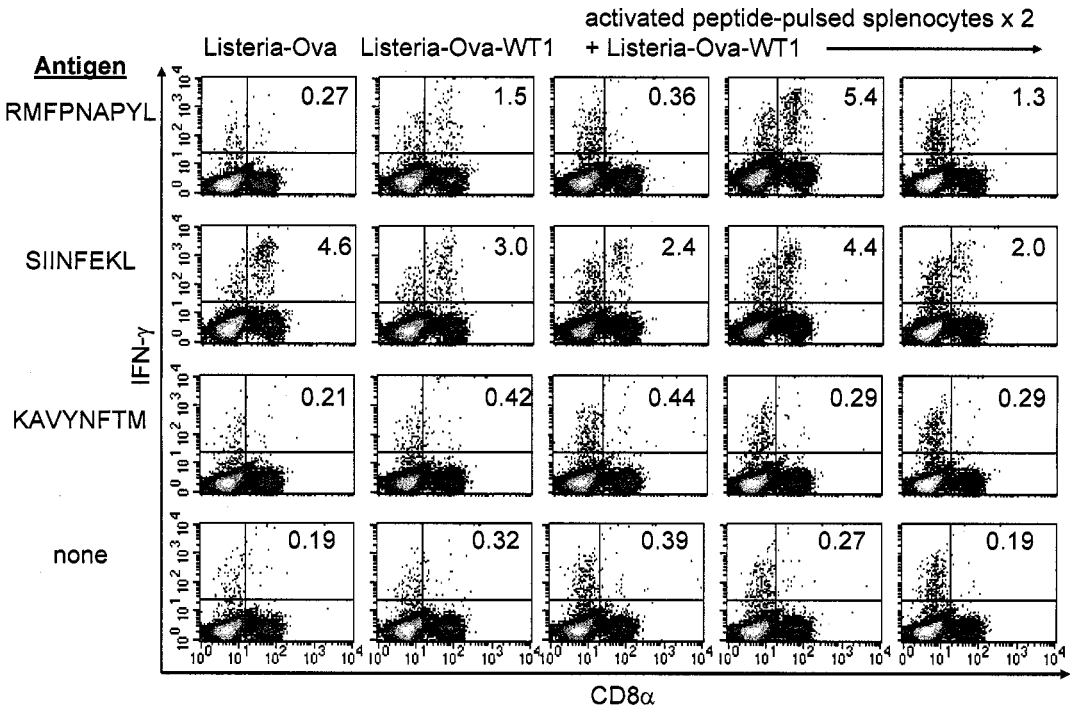

Figure 22: A second generation recombinant WT1-Listeria vector stimulates RMFPNAPYL-specific T cell responses. Mice were immunized twice with RMFPNAPYL peptide-pulsed activated adherent splenocytes and infected 4 weeks after the last immunization with recombinant Listeria expressing RMFPNAPYL (3 right-most columns), only infected once with recombinant Listeria expressing RMFPNAPYL (2^{nd} column from the left), or infected once with a recombinant control Listeria not expressing RMFPNAPYL (leftmost column). Six days post infection, splenocytes were restimulated *in vitro* with RMFPNAPYL, SIINFEKL, KAVYNFTM (negative control) or no peptide for 6 hours in the presence of brefeldin A and analyzed for IFN-γ production by intracellular staining. Cell percentages are % of $CD8^+$ T cells. Each column represents responses from an individual mouse.

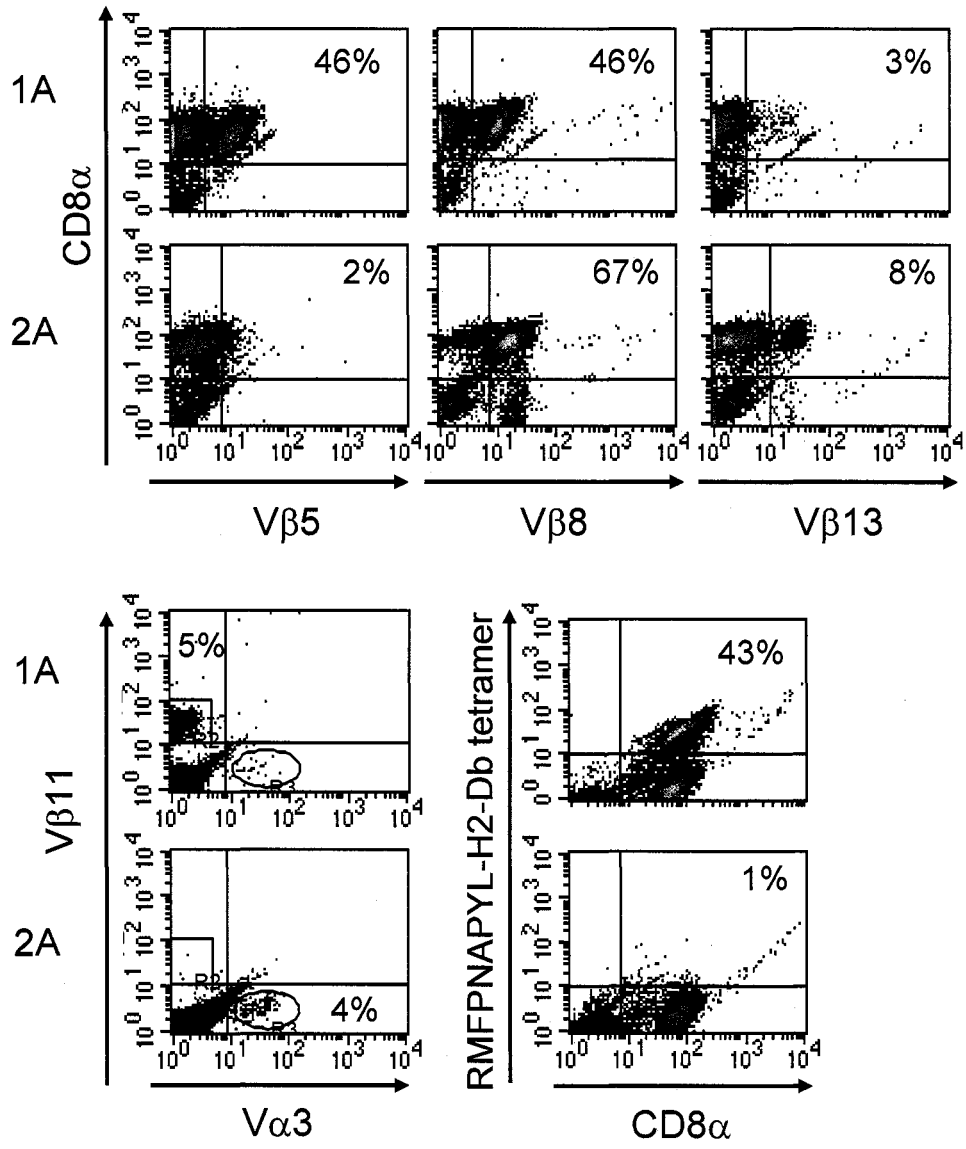

Figure 23: WT1-specific T cell lines 1A and 2A each consist of at least 3 different T cell populations. TCR usage within T cell lines 1A and 2A was assessed by staining with antibodies to TCR Vα and Vβ domains. Tetramer binding was also assessed by incubating cells with PE-labeled RMFPNAPYL-H2-D^b tetramers and antibodies to CD8α.

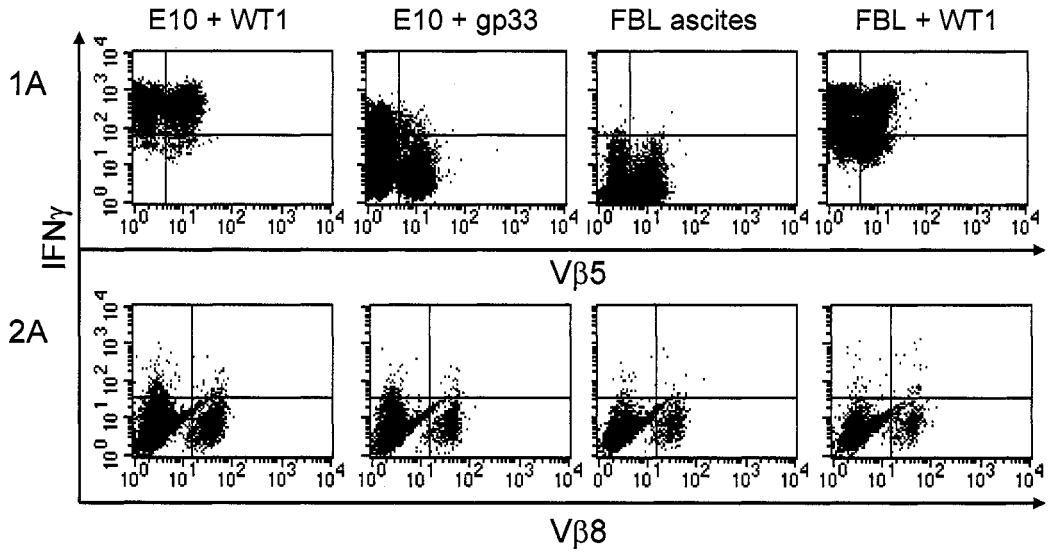

Figure 24: Line 1A produces IFN-γ in response to RMFPNAPYL peptide stimulation while line 2A does not. T cell lines 1A and 2A were incubated with E10 (*in vitro* cultured) or FBL (ascites) cells pulsed with or without RMFPNAPYL peptide for 6 hours in the presence of brefeldin A and analyzed for IFN-γ production by intracellular cytokine staining and TCR Vβ expression.

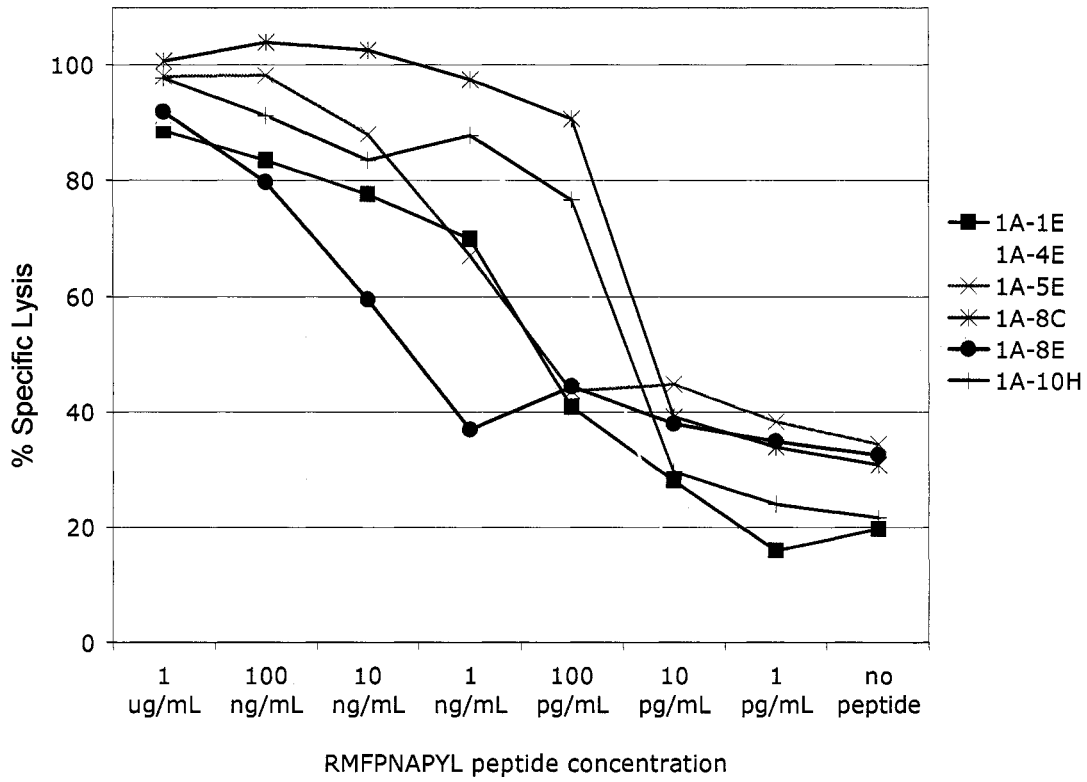

Figure 25: RMFPNAPYL-specific T cell clones isolated from the 1A line exhibit a range of avidities. T cell clones specific for RMFPNAPYL were isolated by limiting dilution and expanded *in vitro*. Avidities of the T cell clones were compared in a 4 hour chromium release assay using RMAS cells pulsed with different concentrations of the RMFPNAPYL peptide at a T cell to target cell ratio of 10:1. A representative peptide titration is shown.

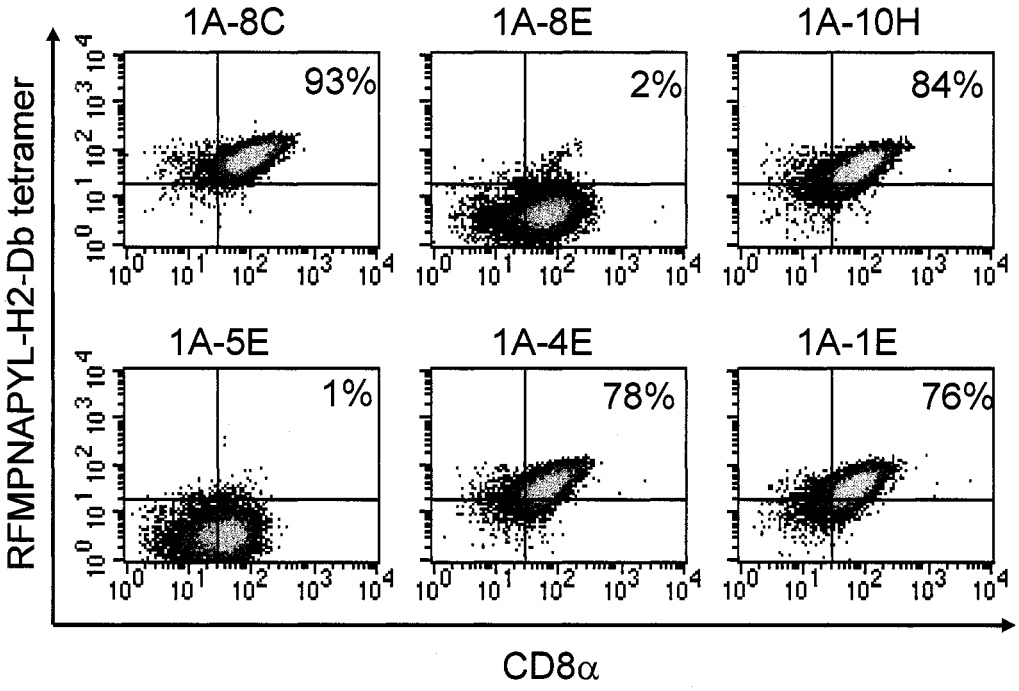

Figure 26: Tetramer staining profiles of the 1A-derived T cell clones. T cell clones were incubated with antibodies to CD8α and RMFPNAPYL-H2-D^b tetramers at 4°C for 30 minutes.

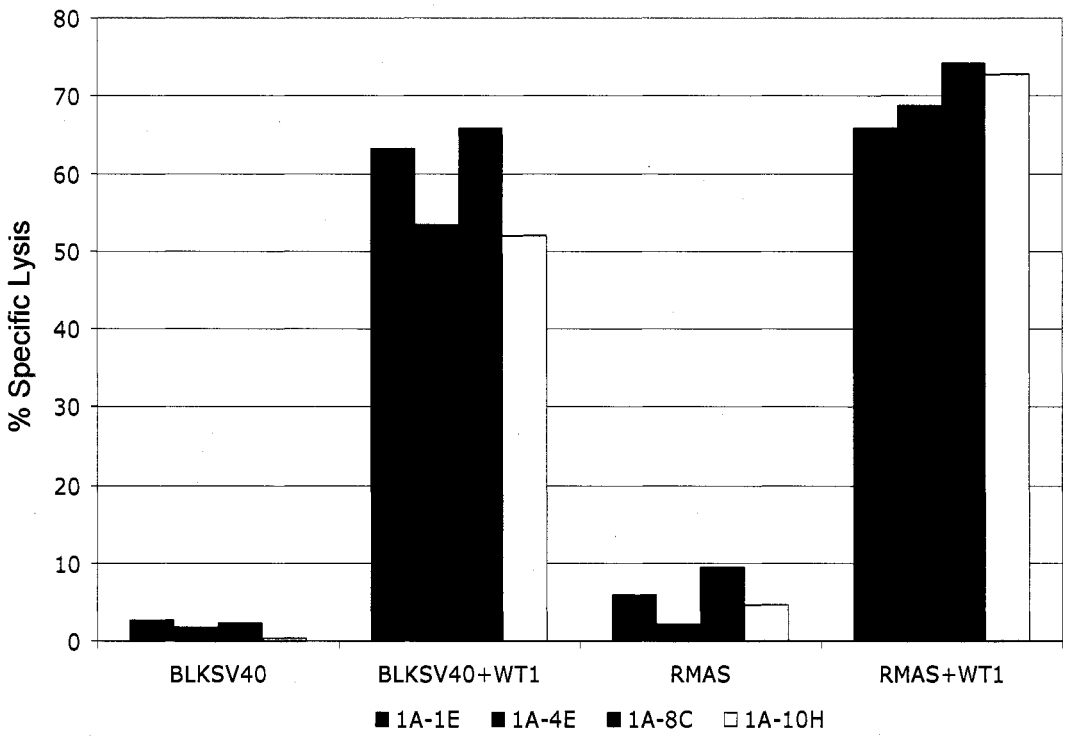

Figure 27: The highest avidity 1A line-derived T cell clones fail to lyse WT1 expressing tumors. The highest avidity T cell clones isolated from the 1A line (1E, 4E, 8C, and 10H) were incubated with ^{51}Cr labeled BLKSV40 or RMAS tumor cells pulsed with or without RMFPNAPYL peptide in a 4 hour chromium release assay at a T cell to tumor cell ratio of 10:1.

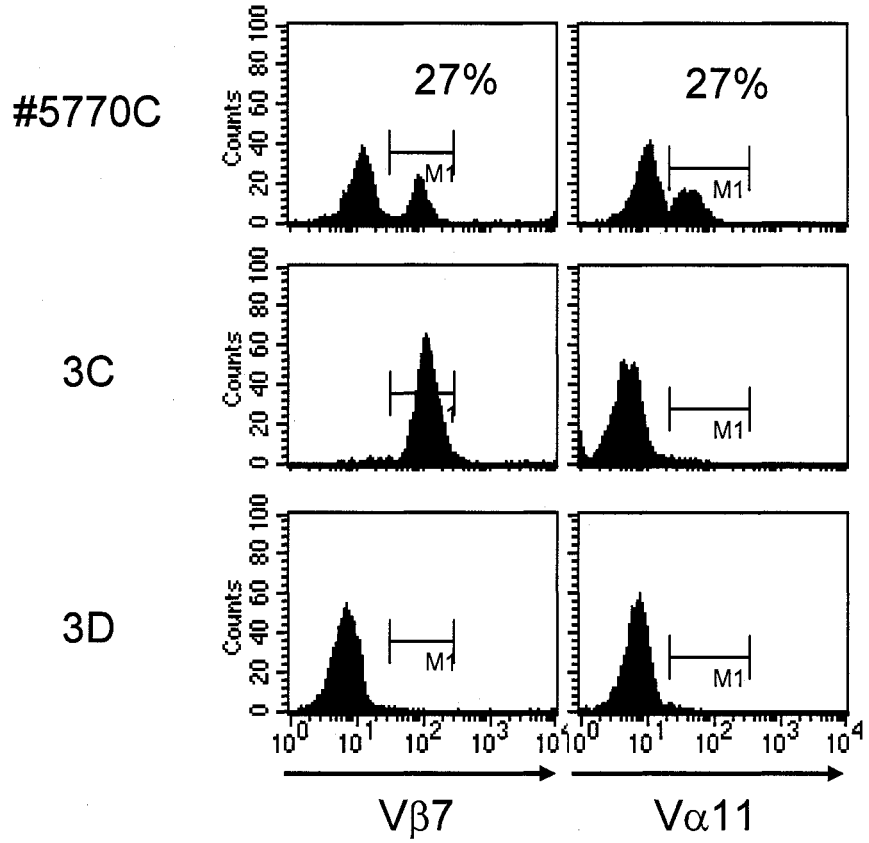

Figure 28: Novel RMFPNAPYL-specific TCRs detected in 3 T cell lines. T cell lines #5770C, 3C, and 3D were stained with antibodies to a variety of different TCR Vα and Vβ domains to assess TCR usage. The Vα11Vβ7$^-$ T cell population within #5770C expresses Vβ8 (not shown). Line 3D also stains negative for Vβ5, 8, 11, 12, 13, and 14 and Vα2 and 3 (not shown).

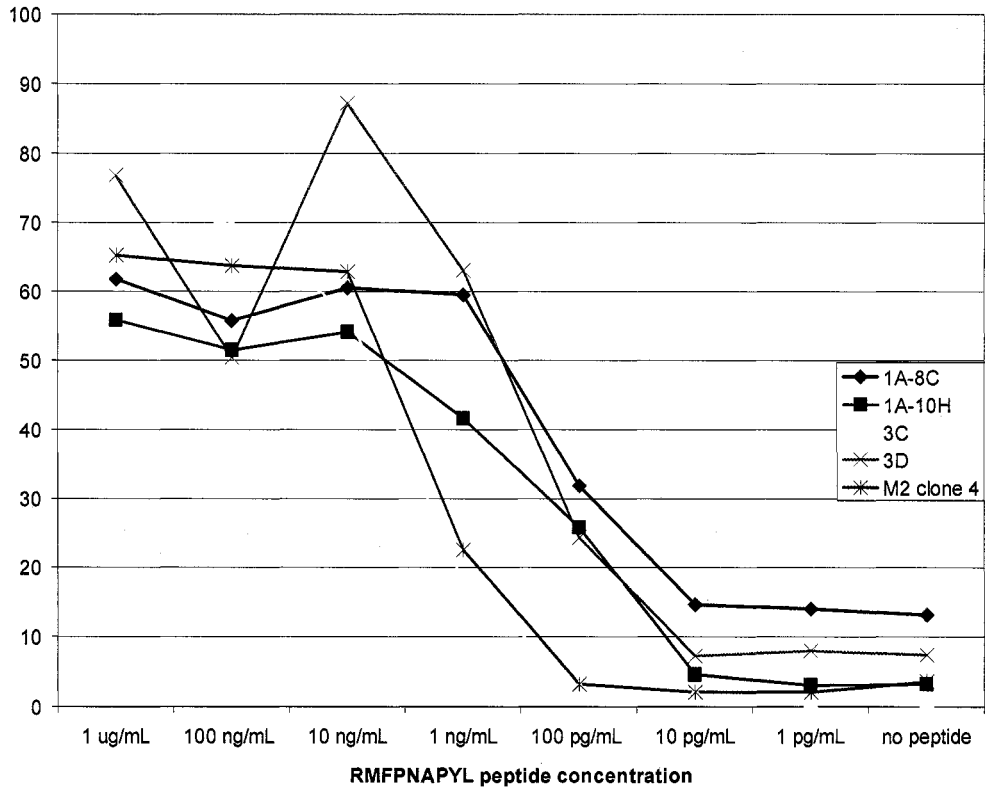

Figure 29: Comparison of the highest avidity T cells specific for RMFPNAPYL isolated from different mice. Avidities of T cell lines and clones specific for RMFPNAPYL were compared in a 4 hour chromium release assay using RMAS cells pulsed with different concentrations of the RMFPNAPYL peptide at a T cell to target cell ratio of 10:1. A representative peptide titration is shown.

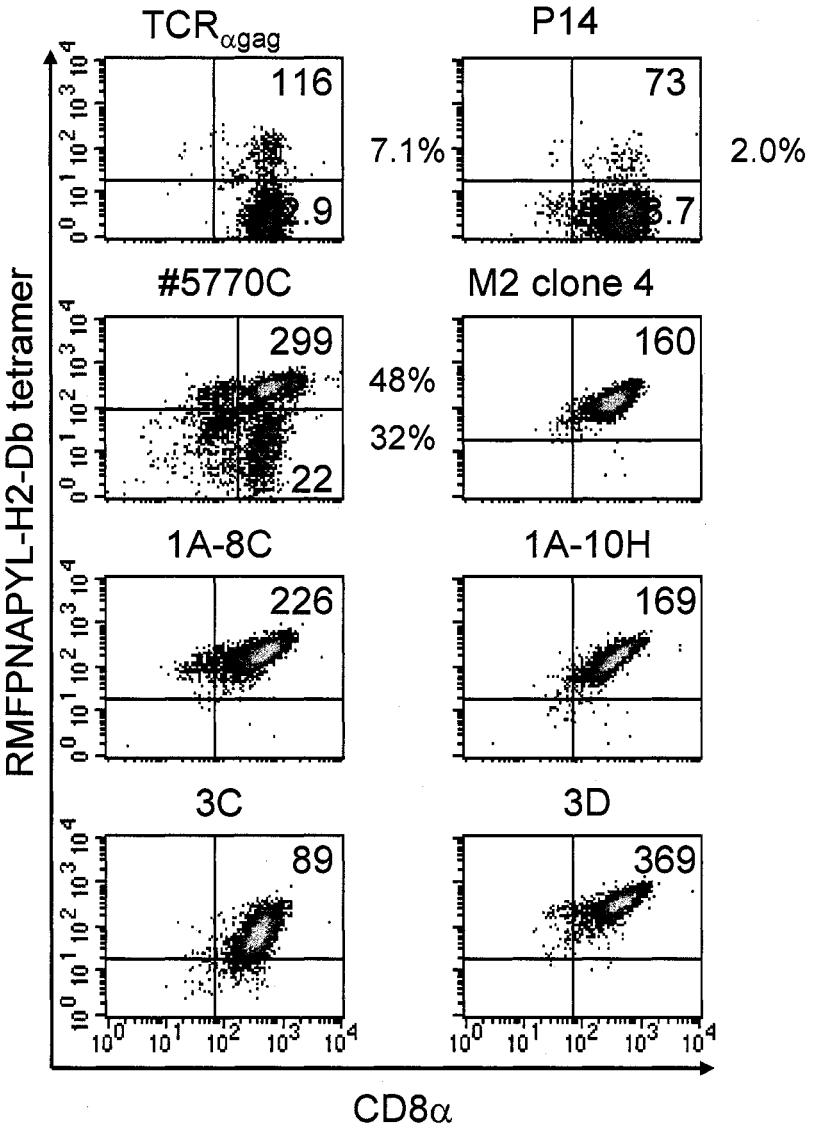

Figure 30: Tetramer staining profiles of the highest avidity T cell lines and clones isolated. T cells were incubated with antibodies to CD8α and RMFPNAPYL-H2-D^b tetramers at 4°C for 30 minutes. MFI of the tetramer signal is indicated in the corners of the plots. For T cell populations that span multiple quadrants, the percentage of T cells within each quadrant is indicated just outside, and to the right, of the plots.

Table 1: The murine T cell response to WT1 is polyclonal and encompasses a range of avidities

Line	TCR Vβ expression	clones	lowest peptide concentration for lysis of RMAS cells	tetramer staining
#3	11	#33	100 ng	negative
M2			1 ng	positive
	5	4, 11	1 ng	positive
	8		1 ng	intermediate
	13		1 ng	low
1A	5	5E, 8E	1 ng, 10 ng	negative
	8	1E, 4E, 8C, 10H	100 pg	positive
	11	9C	100 ng	negative
2A	8, 13			negative
3C	7		1 ng	positive
3D	?		100 pg	positive
#5770C	7, 8			positive/ intermediate

Chapter 4: Isolation and Characterization of Human WT1-specific T Cells

Introduction:

Previous efforts within our laboratory to generate human $CD8^+$ T cells specific for the WT1-derived peptide RMFPNAPYL in the context of HLA-A2 resulted in the isolation of two distinct T cell clones. One of these clones recognized HLA-$A2^+$, WT1-expressing tumors, and the second T cell clone was unable to recognize human tumors expressing WT1. We decided to clone the TCRs expressed by these T cells and use them as templates for *in vitro* engineering of higher affinity TCR mutants using the yeast display system. We also used the higher avidity TCR to examine a novel strategy for promoting preferential pairing of the introduced TCRα and β chains within TCR gene transduced T cells. Mismatched pairing of introduced and endogenous TCRs is common in TCR transduced T cells and not only lowers the effective levels of introduced, tumor-specific TCR on the cell surface, but also poses a potential therapeutic risk because of the creation of novel, unselected TCR pairs.

Materials and Methods:

T cell culture supplements: T cells were cultured in RPMI 1640 supplemented with 25 mM HEPES, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA), 50 μM β-mercaptoethanol (Sigma, St. Louis, MO) and 10% normal human serum (CTL medium). Human serum was isolated in our laboratory from healthy donors after informed consent was obtained. T cell clones, or

primary T cells from PBMC used for retroviral transduction were stimulated to proliferate using the rapid expansion protocol (REP) described by Riddell and Greenberg (120). IL-7 was purchased from R&D Systems (Minneapolis, MN), IL-2 from Chiron (Emeryville, CA) and αCD3 (OKT3, muromonab) was obtained from Ortho Biotec (Bridgewater, NJ).

Generation of T cell clones: To generate clone RS28, $4x10^6$ non-adherent PBMC were stimulated with $0.8x10^6$ irradiated (75 Gy), TAP-deficient T2 cells pulsed with RMFPNAPYL peptide (50μM) and β2 microglobulin (5 μg/ml) in wells of a 24 well plate. Cells received IL-7 (10 ng/mL) on days 1 and 4. On day 7 the cultures were restimulated in the same manner, except IL-2 (10 U/mL) was added on day 2 of the second stimulation. On day 7 following the second stimulation tetramer positive cells were isolated, and then cloned and expanded using the REP protocol. To generate clone CE10, $3x10^6$ CD8 T cells were stimulated with $0.15x10^6$ DC matured over 7 days as described (108) per well of 24 well plates (T cell:DC ratio = 1:20) with 0.5μM free RMFPNAPYL peptide and IL-7 (10 ng/mL on days 1 and 4). Cells were restimulated identically on day 7 except that the T cell:DC ratio was changed to 1:10 and cells received an additional 5 U/mL IL-2 on the second day of the stimulation. Seven days later, cells were restimulated again without IL-7 supplementation. Tetramer positive cells were sorted and cloned ten days following the third stimulation and expanded using the REP protocol.

Antibodies, tetramer, and peptides: Anti-human CD8 and IFN-γ (for ICC) mAb were obtained from BD Biosciences (San Jose, CA) and anti-human TCRVβ13 and Vβ21 mAb from Beckman Coulter (Fullerton, CA). IFNγ ELISPOT capture and detection mAb were from clones 1D1K and 7B61 (Mabtech, Mariemont, OH). HLA-A0201 peptide tetramers were generated as previously described (121) using streptavidin-PE (Molecular Probes, Eugene, OR). WT1 (RMFPNAPYL), HIV-gag (SLYNTVATL), and p53 (LLGRNSFEV) – derived peptides were synthesized and HPLC purified by Synpep (Dublin, CA).

TCRα and β chain identification: The TCR chains expressed by CE10 and RS28 were amplified using a SMART RACE cDNA Amplification Kit (Clontech #K1811-1) and the following primers: CAG CCG CAG CGT CAT GAG CAG ATT A (Cα), CCA CTT CCA GGG CTG CCT TCA GAA ATC (Cβ1), and TGG GAT GGT TTT GGA GCT AGC CTC TGG (Cβ2). PCR products were gel-purified, cloned into pCR2.1 Topo (Invitrogen), and multiple bacterial colonies were sequenced and BLAST searched using the NCBI database to identify TCR chain usage.

Retrovirus construction and transduction: To verify that the identified TCR chains constituted a functional receptor, the TCRα and β chains from clones RS28 and CE10 were individually cloned into the BamHI and NotI sites of the retroviral vector pLZRSpBMN-Z (a kind gift from Dr. Gary Nolan), replacing the LacZ gene. Bacterial plasmid DNA was transfected into Phoenix-Galv packaging cell lines using

lipofectamine and selected with puromycin (1 μg/mL). Retroviral supernatant was harvested from confluent packaging cells incubated overnight at 32°C and concentrated in 11 mL batches by centrifugation in a SW-41Ti rotor (Beckman) at 20,000 rpm for 2 hours at 4°C. Retroviral pellets were resuspended in 0.5 - 1 mL CTL medium and $5x10^6$ PBMC at day 2 post REP were spin-infected in 1 mL of viral supernatant, polybrene (1-2 μg/mL), and IL-2 (50 U/mL) at 1200 rpm and 32°C for 1 hour then placed at 37°C. Cells were washed and resuspended in fresh CTL medium and IL-2 16 hours later.

For TCR pairing studies with the cysteine modified TCRs, the full-length wild type Vα21 and Vβ21 sequences were inserted into the retroviral vector pBullet (kindly provided by Ralph Willemsen) (122), with IRES-puro and -neo cassettes (BD Pharmingen) inserted downstream of the TCR α and β chains, respectively (79, 123). The wild-type Vα21 and Vβ21 TCR chains were modified by mutating residue 48 in the Cα region from thr to cys and residue 57 of the Cβ region from ser to cys using the Quick change XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following primers were used for the α and β chains: αforw: TCT GAT GTG TAT ATC ACA GAC AAA TGT GTG CTA GAC ATG AGG TCT ATG GAC TT; αrev: TGT CTA GCA CAC ATT TGT CTG TGA TAT ACA CAT CAG AAT CCT TAC; βforw: GAG GTG CAC AGT GGG GTC TGC ACA G; βrev CTG TGC AGA CCC CAC TGT GCA CCT C. Packaging cells were transfected using Fugene transfection reagent (Takara, Gennevilliers, France) with pBullet-TCR chain constructs, gag-pol (pHIT60) and env (pCOLT-GALV) vectors (78). Activated human PBMC were

transduced twice in the presence of 40-100 U/ml rhIL-2 and polybrene (Sigma-Aldrich, St. Louis, MO) at 4 μg/ml with packaging cell supernatant containing retroviral TCRα-IRES-puro and TCRβ-IRES-neo constructs, GFP-IRES-neo, or IRES-neo and IRES-puro control vectors. T cells were expanded by weekly stimulation with anti-human-CD3/CD28 Dynabeads^{TM} (1 xl/10^6 cells) (Dynal, Oslo, Norway) and human rhIL-2 (40-100 U/ml) and selected with geneticin (Gibco, Karlsruhe, Germany) at 800 μg/ml and puromycin (Sigma) at 5 μg/ml for 1 week. Polyclonal $CD8^+$ T cells transduced with αβ chains, sorted for Vβ chain surface expression, as well as clonal populations of transduced $CD8^+$ T cells, were expanded *in vitro* using the REP protocol.

Chromium release assays: LCL target cells were incubated at 37°C with ^{51}Cr and titrating concentrations of RMFPNAPYL peptide. After a 4–18 hour incubation, target cells were washed three times, resuspended at a concentration of $5x10^4$/mL and 100 μL of cells aliquotted per well of 96-well plates. Effector CTL were counted, washed and resuspended at a concentration of $5x10^5$/mL, and 100 μL of cells aliquotted per well of 96-well plates (for a 10:1 effector/target ratio). Control wells of target cells alone and target cells with lysis buffer were also included to determine spontaneous and maximum lysis levels. Plates were centrifuged at 200xg for 3 min and incubated at 37°C for 4–6 hours. Thirty microliters of supernatant from each well were assayed for radioactivity using a Lumaplate and TopCount scintillation counter (Perkin Elmer, Boston, MA). Percent specific lysis was calculated as: (average sample

lysis – average spontaneous lysis)/(average total lysis – average spontaneous lysis)x100.

For testing-specific lysis of human $WT1^+$ leukemia cells, leukemic blasts from an $HLA\text{-}A0201^+$ donor were loaded with ^{51}Cr and mixed with T cells at effector/target cell ratios ranging from 30:1 to 0.3:1. To demonstrate specificity of lysis for WT1-expressing targets, "cold" unlabeled T2 cells, pulsed with 10 μM peptide (either WT1-RMFPNAPYL or an irrelevant HLA-A0201-restricted p53 peptide LLGRNSFEV) were added at the initiation of the assay at a cold target to labeled target ratio of 20:1, and the ability of these cold targets to diminish the observed lysis of the leukemic cells assessed.

Assay for intracellular IFN-γ production: T2 cells were pulsed overnight with WT1 or HIV-gag derived peptides (10 μM) at 37°C and the following morning $1x10^6$ cells per well were aliquoted into 96 well plates. $3.3x10^5$ T cells were added per well (E:T = 1:3) and the cells incubated at 37°C. After 2 hours, brefeldin A was added (10 μg/mL final concentration) and the cells incubated at 37°C for an additional 5 hours. Cells were washed, incubated with antibodies to CD8, fixed and permeabilized (cytofix/cytoperm, BD Pharmingen), washed (perm/wash, BD Pharmingen), incubated with mAb to IFN-γ, and analyzed on a flow cytometer.

IFN-γ ELISPOT: T cells transduced with wild-type or cysteine modified TCR chains were sorted for expression of CD8 and equivalent high or intermediate levels of Vβ21

expression. Fifteen thousand T cells were cocultered with 50,000 T2 cells, pulsed with the indicated amount of RMFPNAPYL peptide for 20 hours and assayed for IFN-γ production by ELISPOT as described (124).

Results:

Identification, cloning, and confirmation of WT1-specific TCRs

Two different WT1-specific $CD8^+$ T cell clones (RS28, CE10) were generated from naïve, peripheral T cells of healthy HLA-$A2^+$ donors. Naïve cells were selected as the responding population since it was anticipated that cells that had already encountered WT1 as a self-protein would either be deleted or tolerized.

The avidities of the two clones were compared by analyzing lysis of LCL target cells pulsed with varying concentrations of RMFPNAPYL peptide. The minimum concentration of peptide required for RS28 to lyse LCL target cells was 50 μg/mL, whereas CE10 lysed LCL pulsed with as little as 50 ng/mL RMFPNAPYL peptide (Figure 31; Ho, unpublished data). As tetramer binding is an important quality for TCR templates in the selection of higher affinity TCR mutants using yeast display, the RS28 and CE10 T cell clones were incubated with PE-labeled RMFPNAPYL-HLA-A2 tetramers and staining intensity examined (Figure 32). Compared to normal PBMC as a negative control, RS28 exhibited a roughly one log increase in tetramer binding, and CE10 exhibited a two log increase in tetramer binding. The correlation between the tetramer staining data and the differences in sensitivity to peptide

concentration for target cell lysis suggest that the avidity differences between these two T cell clones predominantly reflects differences in TCR affinity.

CDNAs encoding the TCR chains expressed by these clones were isolated by RACE PCR utilizing TCRα and β chain constant region specific primers. Based on the sequencing of the PCR produccts, we determined that RS28 expresses a Vα4 and Vβ13 TCR while CE10 expresses a Vα21 and Vβ21 TCR. TCRβ chain expression was confirmed by flow cytometry using TCR Vβ-specific antibodies (Figure 32). No commercial antibodies are available for the TCRα chains expressed by these T cell clones.

To verify that the isolated TCR cDNAs constitute functional, WT1-specific TCRs, the respective TCRα and β chain pairs were co-transduced into PBMC from a healthy donor as two separate retroviruses (Vα4 + Vβ13 or Vα21 + Vβ21). Since transduction efficiency for the TCRα chains could not be directly assessed, PBMC were stained with antibodies to CD8 and the appropriate TCR Vβ chain to estimate transduction efficiency (Figure 33a). Approximately 1% of the cultured lymphocytes endogenously expressed both CD8 and Vβ13 or Vβ21. Following transduction, 22% of the cells in culture expressed CD8 and Vβ13 and 15% of the cells expressed CD8 and Vβ21. To determine if RMFPNAPYL antigen recognition was conferred, the transduced PMBC were stained with antibodies to CD8 and a RMFPNAPYL-HLA-A2 tetramer (Figure 33b). In the cells transduced with the RS28-derived Vα4 Vβ13 TCR, 4.8% bound tetramer, and in the cells transduced with the CE10-derived Vα21Vβ21

TCR, almost 1% were tetramer positive. No tetramer positive cells were detected in the mock transduced population. The small percentages of tetramer positive cells likely reflects the low probability of co-transduction with both TCRα and TCRβ chain containing retroviruses as well as low levels of TCR gene expression in some of the transduced T cells.

Transduced T cells were analyzed for IFN-γ production following RMFPNAPYL peptide stimulation. As in the previous experiment, PBMC were transduced with two retroviruses encoding either the RS28-derived Vα4 Vβ13 TCR or the CE10-derived Vα21Vβ21 TCR. Tetramer staining of the transduced cells revealed a slightly lower percentage of tetramer positive cells for both TCR combinations compared to the previous experiment (3% for the RS28 TCR and 0.9% for the CE10 TCR; Figure 34), likely reflecting a decreased viral titer in the supernatants used for transduction. Transduced cells were incubated with the TAP-deficient cell line T2 pulsed with the WT1-derived RMFPNAPYL peptide or an irrelevant HLA-A2 binding HIV gag-derived peptide as a negative control. Cells transduced with WT1-specific TCRs, but not mock transduced T cells, secreted IFN-γ upon stimulation with RMFPNAPYL peptide but not in response to the HIV gag control peptide, demonstrating the ability of these TCRs to specifically confer WT1 antigen recognition to recipient T cells.

A novel strategy for promoting preferential TCRα and β chain pairing

One concern associated with TCR gene therapy is the potential to form mixed TCR dimers in which the introduced and endogenous TCRα and β chains pair with each other (7, 35). Recently, Boulter and colleagues described an approach to stabilize soluble full-length TCRs by engineering a novel disulfide bond between TCRα and β chain constant domains (125). We hypothesized that engineering the mutations that create this novel bond into our WT1-specific TCRs would promote better pairing of the introduced TCR chains with each other and might therefore decrease pairing of the introduced TCR chains with endogenous TCR chains.

To test this hypothesis, we engineered the cysteine mutations creating this novel disulfide bond into the Vα21 and Vβ21 TCR chains expressed by the T cell clone CE10. Wild-type or cysteine modified TCR chains were cloned into the retroviral vector pBullet with an IRES element linking expression of the TCRα chain to a gene encoding puromycin resistance or the TCRβ chain to a gene encoding neomycin resistance. Wild-type or cysteine modified TCRs were transduced into PBMC and selected for one week with both antibiotics. To assess TCR pairing at the cell surface, the transduced and selected T cells were incubated with antibodies to Vβ21 and RMFPNAPYL-HLA-A2 tetramers. The mean fluorescence intensity (MFI) of Vβ21 staining was plotted against the MFI of tetramer staining for both TCRs (Figure 36a, Kuball, et. al., manuscript in preparation). At every level of equivalent Vβ21 expression, T cells expressing cysteine modified TCRs bound more tetramer

than T cells expressing wild-type TCRs, indicating that the modification promoted preferential pairing of the introduced TCR chains with each other.

An increase in appropriate chain pairing should translate into improved functional recognition of antigen. T cells transduced with wild-type or cysteine modified TCRs were sorted into Vβ21 high or Vβ21 intermediate expressing cell populations and assayed for IFN-γ production in an ELISPOT assay using T2 cells pulsed with titrated amounts RMFPNAPYL (Figure 36b, Kuball, et. al., manuscript in preparation). T cells expressing cysteine modified TCRs recognized peptide-pulsed target cells more efficiently than T cells expressing wild-type TCRs, as reflected by a larger number of cells secreting IFN-γ. This difference was particularly striking for T cells expressing intermediate levels of Vβ21. T cells expressing intermediate levels of the cysteine modified TCR exhibited an avidity similar to T cells expressing high levels of the wild-type TCR. In contrast, T cells expressing intermediate levels of the wild-type TCR exhibited poor antigen recognition, even at peptide concentrations two logs higher than that needed to stimulate antigen recognition in the other transduced and sorted T cell populations.

Finally, we asked if the improvements in the functional response to antigen conferred by the cysteine modified TCRs improved transduced T cell recognition of primary leukemic blasts endogenously expressing WT1. PBMC transduced with the wild-type or cysteine modified TCR chains were sorted for equivalent levels of Vβ21 expression and tested for the ability to lyse leukemic blasts from HLA-$A2^{+}$ patients with AML (Figure 39, Kuball, et. al., manuscript in preparation). At an effector to

target ratio of 30:1, T cells expressing cysteine modified TCRs yielded 30-40% specific lysis of AML blasts, while T cells expressing wild type TCRs were unable to lyse AML blasts. Lysis was inhibited by unlabeled T2 cells pulsed with the RMFPNAPYL peptide, but not when T2 cells were pulsed with an irrelevant HLA-A2 binding p53-derived peptide. These results demonstrate that the cysteine modified TCRs, by preferentially promoting proper TCR chain pairing, significantly improve T cell recognition of relevant tumor cell targets compared to T cells expressing wild-type TCRs.

Discussion:

We generated two different WT1-specific T cell clones from the naïve, peripheral repertoire of healthy blood donors. These clones exhibited quite different avidities, with the minimum amount of peptide required for target cell lysis differing by three logs. This avidity difference was reflected in the ability of these clones to recognize tumor cells. CE10 recognized HLA-$A2^{+}$, WT1 expressing tumors, whereas RS28 did not (Ho and Kuball, unpublished data). The difference in avidity between these two clones likely reflects, at least in part, differences in TCR affinity as suggested by three lines of evidence. First, when the clones were incubated with RMFPNAPYL-HLA-A2 tetramers, a log difference in the ability of RS28 and CE10 to bind tetramer was observed. Second, these differences in tetramer binding were maintained following transfer of the respective TCR genes (Kuball, unpublished data) to a polyclonal population of primary T cells. Third, fewer tetramer positive T cells

transduced with the RS28 TCR were able to functionally respond to RMFPNAPYL peptide and produce IFN-γ compared to tetramer positive T cells transduced with the CE10 TCR (Figure 34).

Therefore, we have focused our efforts in creating higher affinity mutants of human WT1-specific TCRs on the CE10-derived Vα21Vβ21 TCR. In addition to using yeast display to select for higher affinity mutants, we are also collaborating with the Strong lab (Fred Hutchinson Cancer Research Center, Seattle, WA) to crystallize the Vα21Vβ21 TCR in complex with RMFPNAPYL-HLA-A2, anticipating that the structural insights yielded will suggest possible specific amino acid residues for mutation to improve the binding interactions between these molecules. Although the CE10 TCR can recognize WT1 positive tumor cells, mutants with higher affinity will be analyzed for improved tumor cell recognition. As has been done with allogeneically generated, WT1-specific TCRs, such high affinity mutants will need to be examined for the ability to recognize normal tissues (14, 69). We anticipate that examination of murine TCRs specific for WT1-RMFPNAPYL in our murine model will yield insights into how much "room" there is for T cells to differentially recognize tumor cells and normal tissues expressing this antigen, guiding our efforts to engineer a human TCR that may prove clinically useful.

The low percentages of tetramer positive and IFN-γ producing cells observed in doubly-transduced (TCRα + TCRβ) T cells in these experiments reflect studies initiated before we developed the retroviral vectors described in Chapter 2, which permit coordinated expression of TCRα and β chains from the same retrovirus. Use

of these vectors should significantly increase the number of T cells expressing both of the transduced TCR chains, facilitating future studies screening high affinity mutants.

TCR transduced T cells containing two αβ TCR pairs can theoretically express up to four different TCRs on the cell surface through mismatched pairing of the endogenous and introduced TCR chains. Most TCR gene therapy studies involve the transduction of polyclonal primary T cells, and thus, one would expect to create a large number of novel, unselected receptors in the transduced T cell population, some of which may recognize self-antigens. Mismatched pairing also decreases the number of correctly paired transduced TCRs on the cell surface, potentially resulting in substantially reduced T cell avidity for the target antigen. TCRα and β chains exhibit a weak affinity for each other (126). Recently, Boulter and colleagues described a simple method to improve this affinity, by mutating a single amino acid residue within both the TCRα and β chain constant domains, resulting in the formation of a novel disulfide bond and permitting the creation of stable, soluble TCRs (80, 125, 127). We engineered these mutations into membrane-bound TCRs and found that they promoted preferential pairing of the mutated, transduced TCR chains. This enhancement in transduced TCR pairing resulted in improved T cell avidity for antigen, particularly for T cells expressing lower levels of surface TCR, and improved tumor cell recognition. Furthermore, this strategy should also enhance the safety of TCR transduced T cells by substantially reducing the number of novel, unselected TCRs on the cell surface.

In summary, we have isolated two human T cell clones that recognize the WT1-derived peptide RMFPNAPYL in HLA-A2. Genetic transfer of these TCRs to primary T cells imparts antigen-specificity, and functional avidity improves when the TCR chains are modified to promote preferential pairing through an engineered novel cysteine bond. Strategies such as these should help maintain parental T cell avidity upon TCR gene transfer to promote efficient recognition and lysis of tumor cells while minimizing the potential for autoimmunity through the creation of mixed TCR dimers.

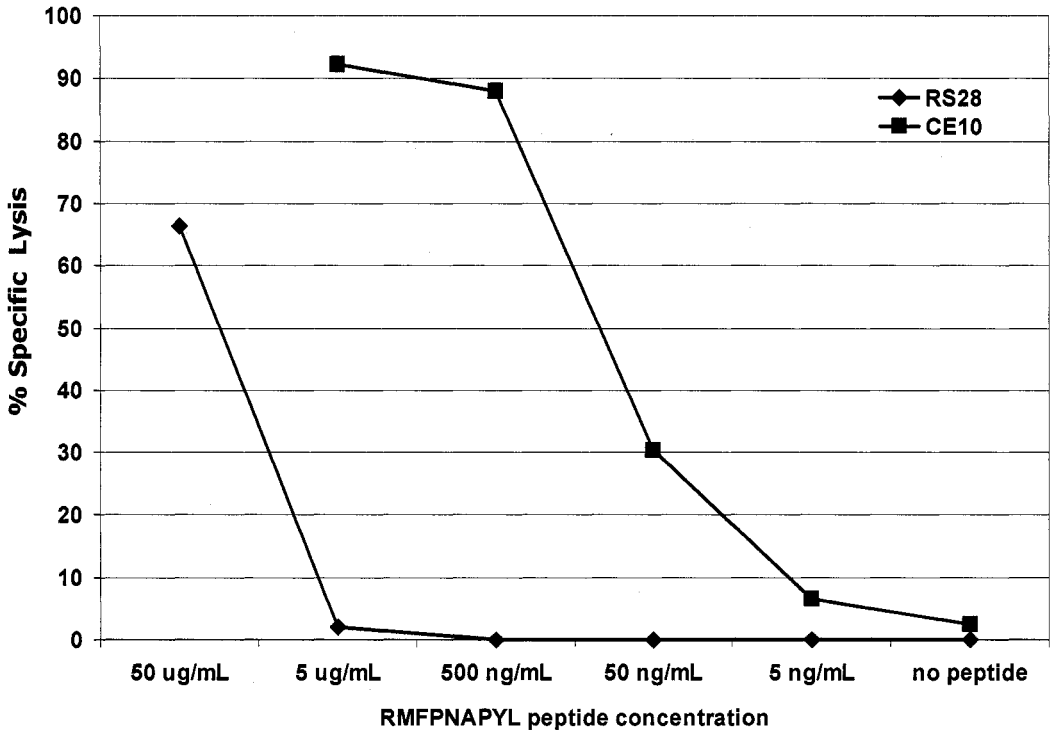

Figure 31: Avidities of the human, WT1-specific T cell clones RS28 and CE10. RS28 and CE10 were incubated with ^{51}Cr-labeled, RMFPNAPYL peptide-pulsed LCL in a 4 hour chromium release assay at a T cell to target cell ratio of 10:1.

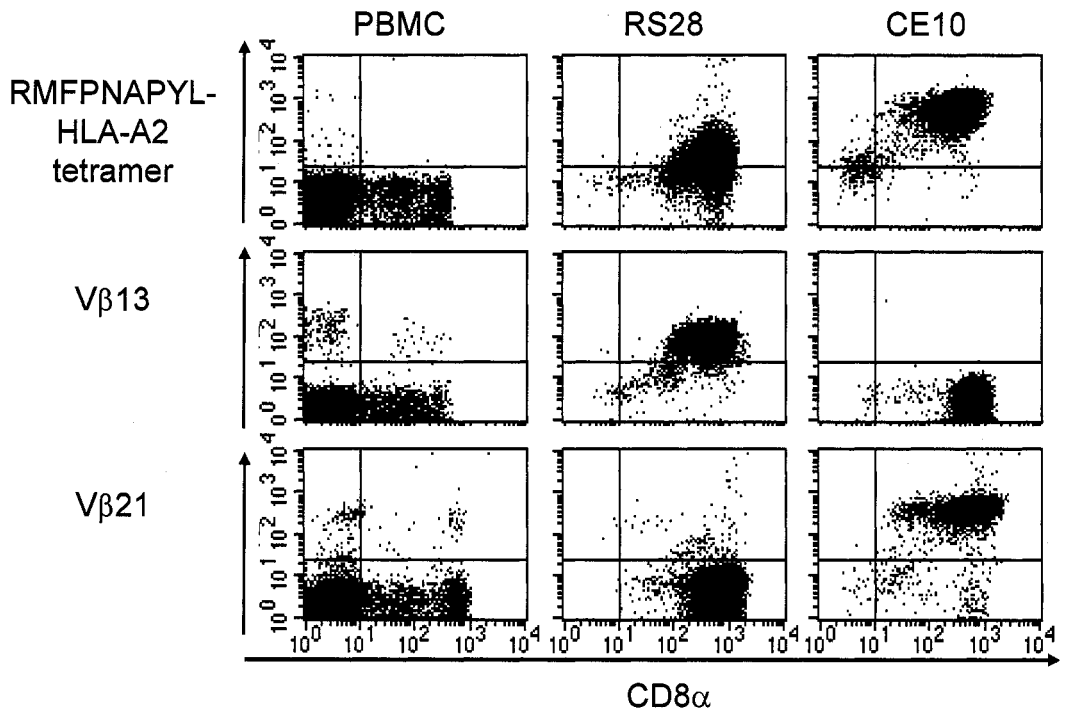

Figure 32: Tetramer binding and TCR Vβ usage of the RS28 and CE10 T cell clones. T cell clones RS28 and CE10 or PBMC from healthy donors were stained with antibodies to CD8 and RMFPNAPYL-HLA-A2 tetramers or antibodies to TCR Vβ13 and Vβ21 domains.

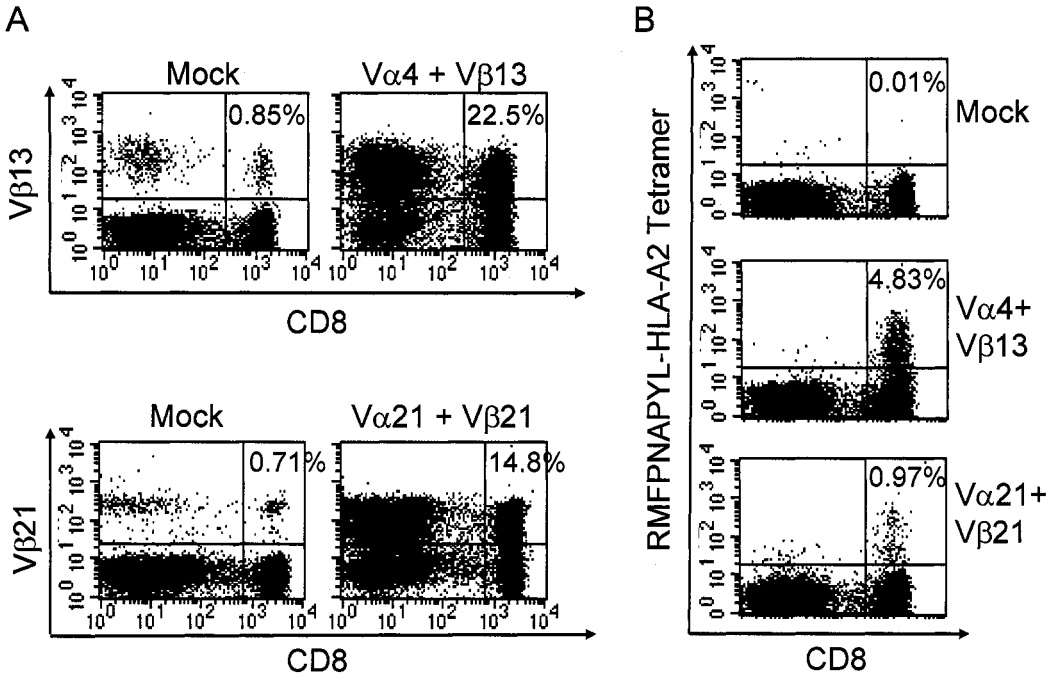

Figure 33: Retroviral transduction of WT1-specific TCRs to PBMC confer the ability to bind RMFPNAPYL-HLA-A2 tetramers. A) PBMC were retrovirally transduced with the RS28-derived TCR chains (Vα4 + Vβ13, each in a separate retrovirus) or with the CE10-derived TCR chains (Vα21 + Vβ21, each in a separate retrovirus) and 10 days later the TCR transduced or mock transduced populations were stained with antibodies to CD8 and the appropriate TCR Vβ chain. B) Mock or TCR transduced PBMC from A were also incubated with antibodies to CD8 and RMFPNAPYL-HLA-A2 tetramers.

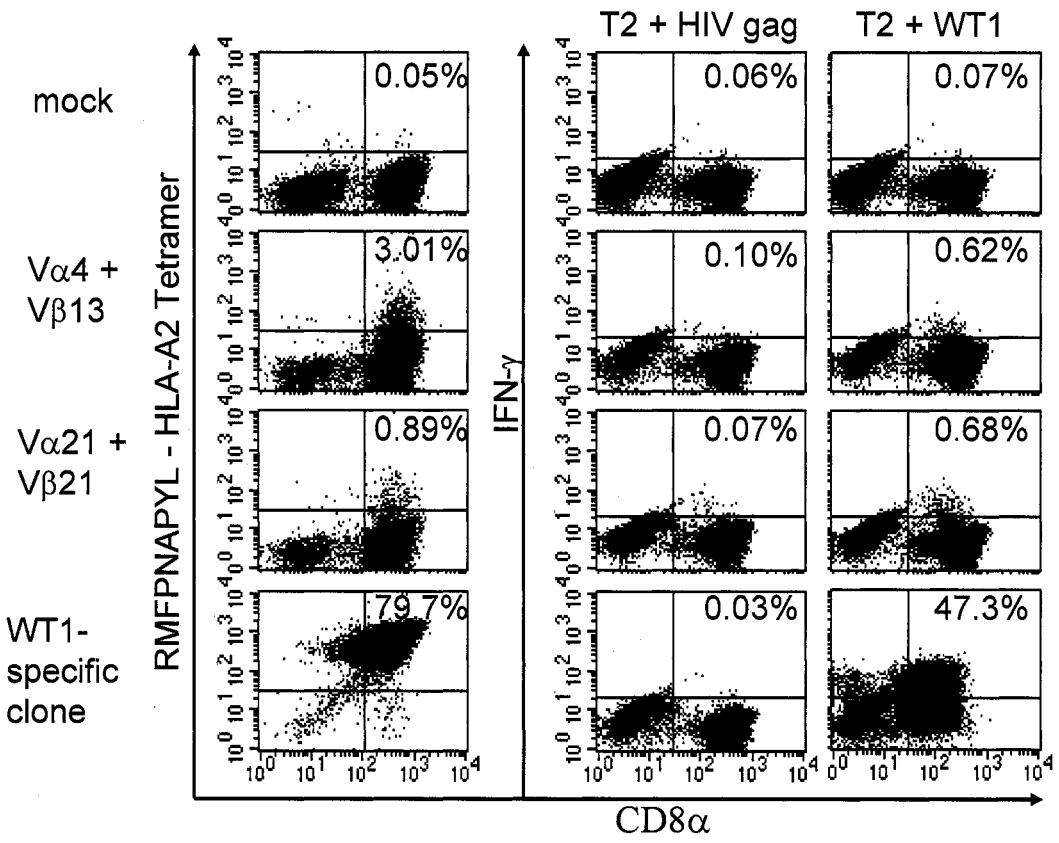

Figure 34: Retroviral transfer of the RS28 and CE10-derived TCR chains confers RMFPNAPYL antigen recognition. PBMC from a healthy donor were stimulated with α-CD3 and IL-2 and transduced with two retroviruses encoding the Va4 and Vb13 or Va21 and Vb21 WT1-specific TCR chains from clones RS28 and CE10, respectively. Transduced cells, mock transduced cells, and a WT1-specific T cell clone were stained with RMFPNAPYL-HLA-A2 tetramers and α-CD8 antibodies or stimulated with T2 cells pulsed with an HLA-A2 binding HIV gag-derived peptide (SLYNTVATL) as a negative control or the WT1-derived peptide RMFPNAPYL for 7 hours in the presence of brefeldin A and stained with antibodies to CD8 and IFN-γ.

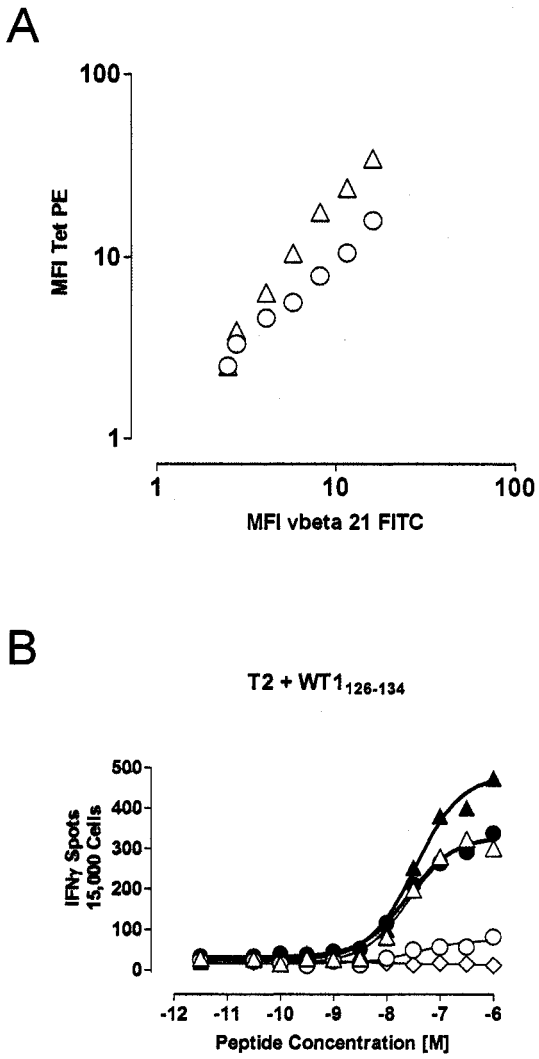

Figure 35: Cysteine modified TCRs increase levels of appropriately paired TCRs at the cell surface and improve avidity for antigen. A) T cells transduced with the Vα21Vβ21 wild-type TCR chains (○) or Vα21Vβ21 cysteine modified TCR chains (△) were simultaneously stained with RMFPNAPYL-HLA-A2 tetramers and α-Vβ21 antibodies. The MFI of the α-Vβ21 signal was plotted against the MFI of the tetramer signal. B) T cells mock transduced (◇) or transduced with Vα21Vβ21 wild-type TCR chains (●,○) or Vα21Vβ21 cysteine modified TCR chains (▲,△) were sorted for lower (open symbols) and higher (closed symbols) levels of Vβ21 TCR expression and incubated with RMFPNAPYL peptide-pulsed T2 cells and analyzed in an IFNγ ELISPOT assay.

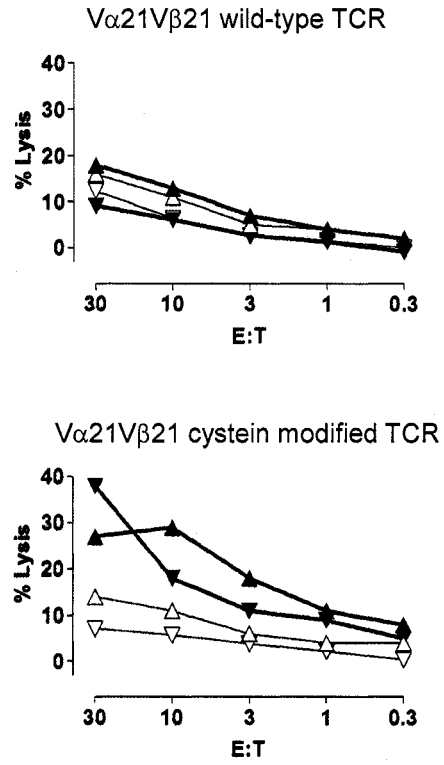

Figure 36: Improved TCR chain pairing leads to improved lysis of leukemic blasts. Wild-type and cysteine modified Vβ21 low TCR expressing T cells from Figure 38B were analyzed for the recognition of two different AML samples (▲,△ and ▼,▽) in a 5-hour ^{51}Chromium release assay at the indicated T cell effector to tumor target cell ratios (E:T). Target cells were co-incubated with an excess (20:1) of cold T2 cells pulsed with the relevant RMFPNAPYL (open symbols, thin lines) or irrelevant peptide (closed symbols, bold lines).

Chapter 5: Expression of Murine and Human TCRs on the Surface of Yeast for Affinity Selection

Introduction:

To generate murine and human TCRs with a higher affinity for the $WT1_{RMFPNAPYL}$ epitope than we could isolate from endogenous T cell repertoires, we have collaborated with the Kranz lab (University of Illinois, Urbana-Champaign) to use PCR-generated mutation, yeast display, and tetramer-based cell sorting to *in vitro* engineer mutants using our cloned WT1-specific TCRs as templates. Single-chain TCRs were constructed and randomly mutated to improve TCR expression on the surface of yeast. TCRs selected for improved yeast surface display were used as templates to create libraries of TCR CDR3α mutants for tetramer-based screening. Since the wild-type murine clone #33 TCR did not have a sufficient affinity to bind RMFPNAPYL-H2-D^b tetramers, we developed a novel strategy for affinity selection using peptide-pulsed APC and density centrifugation.

Materials and Methods:

Antibodies, peptides, peptide-MHC multimers: The WT1-derived peptide RMFPNAPYL was synthesized and HPLC purified by Synpep (Dublin, CA). The QL9 (QLSPFPFDL) peptide recognized by the 2C TCR was synthesized at the Macromolecular Core Facility at Pennsylvania State University and HPLC purified. Position 5 variants of the QL9 peptide (R5 [QLSPRPFDL], H5 [QLSPHPFDL], and M5 [QLSPMPFDL]) were synthesized at the Protein Sciences Facility at the

University of Illinois. Vα3 and Vβ11 antibodies were purchased from BD Pharmingen (San Diego, CA), α-HA antibodies from Boehringer Mannheim (Indianapolis, IN), α-c-myc antibodies from Molecular Probes (Invitrogen, Carlsbad, CA), and Vβ8.2 antibodies were purified from ascites. PE-labeled RMFPNAPYL-H2-D^b tetramers were purchased from Immunomics (Beckman Coulter, San Diego, CA) and APC-labeled RMFPNAPYL-HLA-A2 pentamers were purchased from ProImmune (Springfield, VA). The QL9/L^d-Ig dimers were produced and purified as described (128).

TCR cloning and expression: scTCRs were cloned into the yeast display plasmid pCT302 (129) fused to a gene encoding the yeast surface protein AGA2 and flanked by HA and c-myc epitope tags to monitor surface expression. Expression of the AGA2-scTCR fusion gene was induced by transferring EBY100 yeast cells growing in SD-CAA (2% w/v dextrose, 0.67% w/v yeast nitrogen base, 1% w/v Casamino acids) to galactose-containing media and shaking at 20°C for at least 24 hours. Yeast libraries were constructed as described (82). The 2C-m6 TCR was engineered using yeast display and FACS and has been shown to have an affinity for QL9/L^d of 6 nM (34, 82). C18-1 is a surface stabilized scTCR mutant (Kranz, et. al., unpublished data) of the murine C18 TCR that recognizes a peptide derived from a mutated MAP kinase presented by H2-K^d MHC (130).

Density centrifugation: Induced yeast cells (~$1x10^6$) were aliquoted into tubes that contained $1x10^6$ peptide-loaded T2 cells. A separate aliquot of induced yeast served as a 'pre-centrifugation' control. The yeast/T2 mixtures were allowed to rock at room temperature for 1 hour and then layered onto three mL of Ficoll-Paque PLUS (Pharmacia) in 15 ml conical tubes. After the cells had settled for 10 minutes at room temperature, the tubes were centrifuged at 1500 rpm (~400xg) at 4°C for 30 minutes. After centrifugation, the visible layer of cells above the Ficoll-Paque (the 'interface') was removed from each tube in a 1 mL volume. In some cases, this interface was returned to the rocker for another 30 minute incubation, and the procedure was repeated (centrifugation for 15 min in this 'second centrifugation'). Fold enrichment was calculated as: $(\#\ positive_{final} / total_{final})/(\#\ positive_{initial} / total_{initial})$.

Plasmid rescue and sequencing: DNA encoding the mutant scTCRs was rescued from selected yeast clones using a Zymoprep I Yeast Plasmid Minipreparation Kit (Zymo Research, Orange, CA). Rescued plasmids were introduced into the E. coli strain DH10B (Invitrogen, Carlsbad, CA) by electroporation and were purified from E. coli using a QIAprep Spin Mini-Prep Kit (Qiagen, Valencia, CA) and sequenced at the DNA Core Sequencing Facility at the University of Illinois.

Results:

Evolution of a murine, WT1-specific TCR that can be stably expressed on the surface of yeast

The first WT1-specific TCR we cloned that was available for affinity selection was the murine Vα3Vβ11 TCR expressed by clone #33 (Chapter 3). To express TCR chains on the surface of yeast, single-chain TCRs (scTCRs) were constructed and cloned into the pCT302 vector such that the scTCR was fused to the carboxy-terminus of the yeast surface protein AGA2 and flanked by HA and c-myc epitope tags to monitor expression (AGA2-HA-scTCR-c-myc). Three different constructs were created to determine the optimal orientation for scTCR surface expression: 1) HA-V_β-linker-V_α-c-myc, 2) HA-V_α-linker-V_β-c-myc, and 3) HA-V_α-linker-$V_\beta C_\beta$-c-myc. Yeast transformed with these constructs were incubated with antibodies to HA, c-myc, Vα3, and Vβ11 to assess cell surface expression of these epitopes. Construct 1 only bound the HA antibody, while constructs 2 and 3 bound both HA and c-myc antibodies. None of the constructs bound the TCR Vα3 and Vβ11 antibodies (Richman, unpublished data). These results suggested that, for the latter two constructs, the entire fusion protein was on the cell surface, but sufficiently misfolded to prevent TCR antibody recognition.

A previous study suggested that mutation of TCR framework regions can improve yeast surface expression of TCRs (131). Constructs 2 and 3 were randomly mutated by error-prone PCR and sorted with anti-TCR antibodies. A sort of mutants from construct 2 (HA-V_α-linker-V_β-c-myc) yielded a scTCR, named mWT-3,

containing 6 amino acid substitutions (2 in Vβ11 and 4 in Vα3), that demonstrated improved Vα3 and Vβ11 antibody binding (Figures 37 and 38; Richman, unpublished data). Studies with the 2C TCR had demonstrated enhanced TCR stability and improved surface expression following mutation of leucine 43 of the TCRα chain to proline to create a more favorable interaction with leucine 43 of the TCRβ chain. We engineered this mutation into mWT-3 to create the mWT-3(L43P) mutant and observed further enhancement in Vα3 and Vβ11 antibody binding. To isolate scTCR mutants with additional improvements in anti-TCR antibody binding (and presumably further enhanced stability), the mWT-3(L43P) mutant was used as a template for a second round of error-prone mutagenesis and eight additional unique mutants with improved stability were isolated. TCR Vα3 and Vβ11 antibody staining of one of these mutants, mWT1-B7, is shown with its predecessors in Figure 38.

MWT1-B7 and several related scTCRs were used as templates for mutant TCR library generation. Based on structural studies of TCR molecules bound to peptide-MHC and data from the 2C TCR (88) two libraries with random mutations within the CDR3α region (encompassing amino acids SIPNR) were created, the second library incorporating an additional error-prone PCR step. In addition, based on another study, a library with random mutations within CDR1α, followed by error-prone PCR, was also created (128). These three libraries were pooled, expressed in yeast, and screened with RMFPNAPYL-H2-D^b tetramers to identify high affinity mutants. Seven separate sorts involving sequential rounds of tetramer staining, fluorescent cell sorting, and

yeast recovery failed to yield any mutants that specifically bound RMFPNAPYL-H2-D^b tetramers.

Development of a novel, non-tetramer-based approach for selecting high affinity TCR mutants.

When these mutant scTCR libraries were initially screened, we had not yet identified any murine T cells from immunized mice that bound to tetramer, and therefore could not determine if our tetramer reagent was functional. Accordingly, we set out to develop a novel method to screen TCR libraries for higher affinity mutants without requiring the use of fluorescently-labeled peptide-MHC multimers or clonotypic antibodies. Shusta and colleagues (132) had demonstrated that yeast expressing high affinity TCRs can bind to the TAP-deficient cell line T2 when it is pulsed with the appropriate peptide and visualized as "rosettes" under a microscope. We took advantage of this finding and the density differential between lymphoid cells and yeast to devise a separation strategy based on density centrifugation (119). Yeast expressing scTCRs were incubated with peptide-pulsed T2 cells, layered onto Ficoll-Paque, and centrifuged at 1500 rpm for 30 minutes. Yeast cells that do not recognize, or have a low affinity for, the peptide-MHC on the surface of T2 cells formed a pellet at the bottom, whereas yeast cells that formed stable conjugates with the peptide-pulsed T2 cells were retained at the interface. Subsequent re-incubation and centrifugation of the recovered yeast with peptide-pulsed T2 cells further enriched for high affinity TCRs.

To determine the receptor-ligand affinity range that can be selected using this strategy, we examined enrichment of the high affinity mutant of the 2C TCR, 2C-m6, spiked at a ratio of 1:1000 within an excess of non-binding C18-1 TCR expressing yeast. Yeast cells were incubated with T2-L^d cells pulsed with one of three different 2C TCR peptide variants, M5, H5, or R5 that when bound to L^d have affinities for 2C-m6 of 34 nM, 78 nM, and >1 μM, respectively (34). Yeast samples pre- and post-centrifugation were incubated with antibodies to the 2C-m6 and C18-1 TCR chains, analyzed by flow cytometry, and the ratios of the two populations of yeast with respect to one another for each of the peptide selections were calculated (Figure 39a, (119)). A single density centrifugation resulted in dramatic enrichment of yeast expressing the 2C-m6 TCR for the M5 (970 fold) and H5 (950 fold) ligands. However, for the lower affinity R5 ligand, enrichment was barely detectable in one of two replicate experiments. However, a second incubation of these selected yeast cells with T2 cells pulsed with the R5 ligand resulted in greater enrichment (Figure 39b). These results demonstrate two important findings. First, high level enrichment, on the order of 1000-fold, can be achieved for TCR-peptide-MHC interactions with affinities greater than 100 nM with just a single centrifugation. Second, the higher the affinity of the TCR-peptide-MHC interaction, the greater the enrichment observed. Both of these features are desirable for selecting high affinity TCRs.

To determine if density centrifugation can isolate high affinity TCRs from a mutated TCR library, a 2C TCR CDR3α library from which high affinity TCRs were previously isolated (82) was incubated with T2-L^d cells pulsed with the QL9 peptide

and subjected to two sequential rounds of density centrifugation. Yeast cells that remained in the interface were plated and the relative affinities of the TCRs from individual colonies examined. Yeast clones expressing the selected TCR mutants, the previously isolated and described 2C-m6 mutant, or the stabilized version of the wild-type 2C TCR (2C-T7) were incubated with antibodies to the 2C TCRβ chain or soluble QL9-L^d dimers and analyzed by flow cytometry. All of the yeast clones expressed roughly equivalent levels of surface TCR. Compared to the wild-type 2C-T7 TCR, QL9-L^d dimers bound to each of the mutants more strongly (Figure 40), and QL9-L^d binding correlated with yeast recovery following density centrifugation (119).

Based on the success of the density centrifugation approach in isolating high affinity 2C TCR mutants, we re-screened the mWT1-B7 and related template libraries using this approach. Despite seven separate attempts, we were still unable to isolate higher affinity mutants.

Evolution of a human, WT1-specific TCR that can be stably expressed on the surface of yeast

We have also begun generating high affinity human TCRs using the yeast display system. Single-chain TCRs from the RS28-derived Vα4Vβ13 receptor and CE10-derived Vα21Vβ21 receptor were made as both Vα-linker-Vβ and Vβ-linker-Vα constructs. Yeast cells expressing these constructs were incubated with antibodies to HA, c-myc, and Vβ13 or Vβ21 and analyzed on a flow cytometer. Both configurations for both TCRs bound α-HA antibodies specific for the HA linker on

the amino terminus of the scTCR, however none of the constructs bound the respective anti-Vβ antibodies and only the RS28 constructs bound anti-c-myc antibodies (Richman, unpublished data).

To create stabilized versions of these receptors, with framework region mutations, that could be used as templates for affinity maturation, four libraries (one for each construct) of random mutations within the scTCRs were created and sorted for Vβ and c-myc expression. Three different mutants of the CE10 Vβ-linker-Vα construct were isolated that exhibited enhanced binding to the Vβ21 and c-myc antibodies (Figure 44; Richman, unpublished data). However, staining for c-myc expression was still one log lower than that for the stabilized mWT1-B7 TCR. TCRα chain expression for these mutants could not be assessed as commercial antibodies to those receptor chains are not available. Although the CE10 TCR expressed in T cells binds to RMFPNAPYL-HLA-A2 tetramers, the stabilized scTCR does not. It is formally possible that the framework region mutations affect antigen binding, but a lack of tetramer binding to wild-type TCRs is frequently noted in the yeast display system, even when the parental T cell clone bound tetramer, likely because of differences in yeast and mammalian cell membranes. CDR3α mutant libraries (~10^7 mutants in each) were made by PCR with degenerate primers using all three of the stabilized CE10 receptors as templates and mixed in a 1:1:1 ratio for screening. Yeast cells were incubated with antibodies to c-myc and RMFPNAPYL-HLA-A2 pentamers and analyzed by flow cytometry. Four separate screens have thus far failed to identify any mutants that resulted in binding to these multimers.

Discussion:

As with previous TCRs engineered in the yeast display system (89, 132), both the murine and human WT1-specific scTCRs could not be stably expressed, without further changes, on the surface of yeast. Using random mutagenesis across the entire scTCR, we engineered stabilized versions of both the murine and human TCRs that bound available TCR V region antibodies as well as antibodies recognizing the flanking HA and c-myc epitope tags. Again, as commonly occurs with wild-type scTCRs in the yeast display system, none of the stabilized mutants bound to the appropriate peptide-MHC multimers. Typically, the affinity (Kd) of the TCR for peptide-MHC must be greater than 1 μM to observe tetramer binding to yeast, a value which is at the high end of wild-type TCR affinities. Even the surface stabilized version of the 2C TCR, for which a number of high affinity mutants were successfully generated, does not bind to appropriate tetramers when expressed on yeast (82, 88).

Despite multiple screens of the mutant scTCR libraries, we have been unable, thus far, to identify murine or human TCRs specific for WT1 with sufficiently higher affinity to bind tetramers. There are several possible explanations for these results. For the murine clone #33-derived TCR, in particular, the starting affinity may have been so low that mutations creating affinity improvements of even 1000 fold may have bound tetramer poorly when expressed on yeast. Alternatively, the framework region mutations engineered to stabilize murine and human scTCR expression on the surface of yeast may have interfered with the conformation of the antigen binding site. A

third possible explanation is that we have targeted the wrong region of the TCR for mutation. Most of the available structural data on TCR-peptide-MHC binding suggests that the CDR3α and CDR3β regions of the TCR contribute the most to peptide recognition, although exceptions have been described (133). Based on work with the 2C TCR, we focused most of the mutations in our scTCR libraries on the CDR3α region, however, without structural data for these TCRs, it is difficult to predict *a priori* which region of the TCR contributes the most to peptide recognition, and further mutations will be pursued.

In addition to screening the mutant scTCR libraries with fluorescently-labeled peptide-MHC multimers, we also developed a novel selection strategy based on binding to peptide-pulsed APC and density centrifugation. This strategy successfully isolated high affinity mutants from a stabilized 2C TCR library, but again did not yield any mutants when used to screen the murine libraries. The same possible reasons for failure described above apply here as well. In fact, based on the observed sensitivity of tetramer binding (~1 μM) in this system compared to the observed sensitivity of density centrifugation (~ 100 nM), peptide-MHC multimer staining may have a somewhat lower threshold for detecting improved mutants than density centrifugation, although we have not tested this hypothesis rigorously. One major advantage of density centrifugation over multimer incubation is that it obviates the need to express and purify individual MHC molecules in complex with peptide. Thus, density centrifugation should enable the generation and selection of high affinity TCRs

specific for a number of MHC molecules that do not reproducibly refold well to make tetramers.

Based upon our experiences and the reagents we currently have available, we have decided to try generating high affinity, murine TCR mutants using some of our more recently isolated WT1-specific murine T cell clones that have avidities three logs higher than the clone #33-derived TCR. As initial TCR affinity may dictate the final affinity that can be reached, we hope that by starting out with a higher affinity TCR we will be more likely to isolate mutants that can mediate recognition of WT1-expressing tumors. For the human, WT1-specific TCRs, we are currently developing a system to express full-length TCRs on the surface of yeast instead of scTCRs. TCRs containing the constant domain may exhibit greater stability (126), and require fewer framework region mutations, than scTCRs, particularly if mutated to create a novel disulfide bond between the chains (125). We will use these full-length Vα21Vβ21 TCRs to make CDR3α and CDR3β mutant libraries to screen for higher affinity mutants.

Eventually, the high affinity murine and human TCRs isolated by these approaches will be transduced into primary T cells and analyzed in comparison with the respective wild-type TCRs for the ability to recognize WT1 expressing tumors, as well as normal tissues expressing low levels of WT1, to identify TCRs that mediate differential recognition of these cell types and that may be useful for adoptive immunotherapy. In addition, the kinetic and thermodynamic interactions of these TCRs with the respective peptide-MHC complexes will also be examined to gain

insights into how the biophysical properties of a TCR translate into biological responses. The insights gained from these studies will not only enhance our understanding of how TCRs mediate antigen recognition, but may also lead to the development of clinically useful therapies.

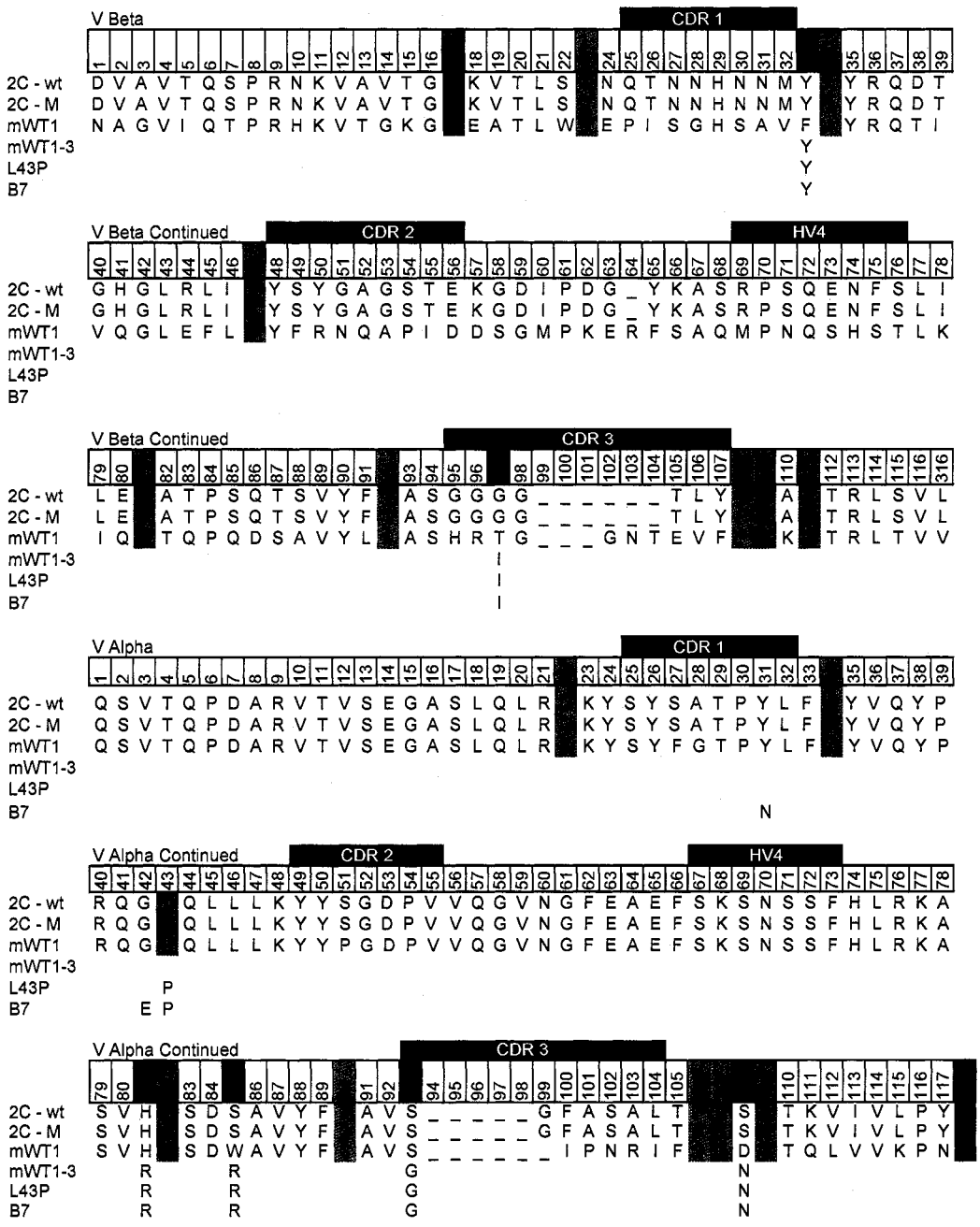

Figure 37: Evolution of scTCR mutations to improve stability and expression on yeast. Alignment of the TCR Vα and Vβ sequences from the wild-type 2C TCR (2C-wt), the surface stabilized version of the 2C TCR (2C-M), the wild-type clone #33-derived TCR (mWT1), the first generation mutant of the WT1 TCR with improved surface stability (mWT1-3), the mWT1-3 mutant with the leucine to proline mutation at position 43 (L43P), and the mWT1-B7 mutant derived from a second round of random mutagenesis across the mWT1-3(L43P) scTCR.

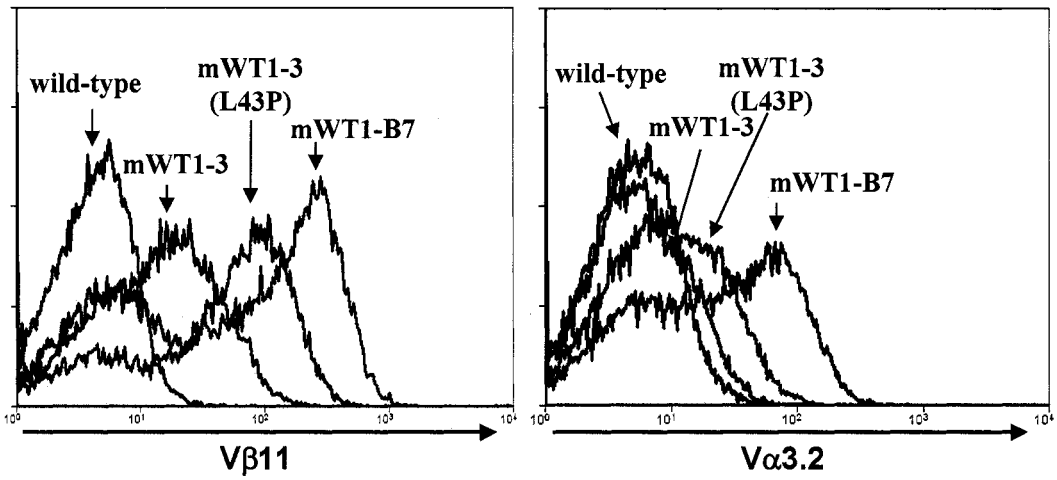

Figure 38: Evolution of mutations within the clone #33-derived TCR improving yeast surface expression. Yeast expressing scTCRs of the original, wild-type Vα3Vβ11 TCR, the mWT1-3 mutant, the mWT1-3 mutant with leucine 43 of Vα3 mutated to proline, and the mWT1-B7 mutant were incubated with antibodies to the Vα3 and Vβ11 TCR domains. In a homogenous population of yeast expressing a single TCR, a negative staining population is always seen and serves as an internal control.

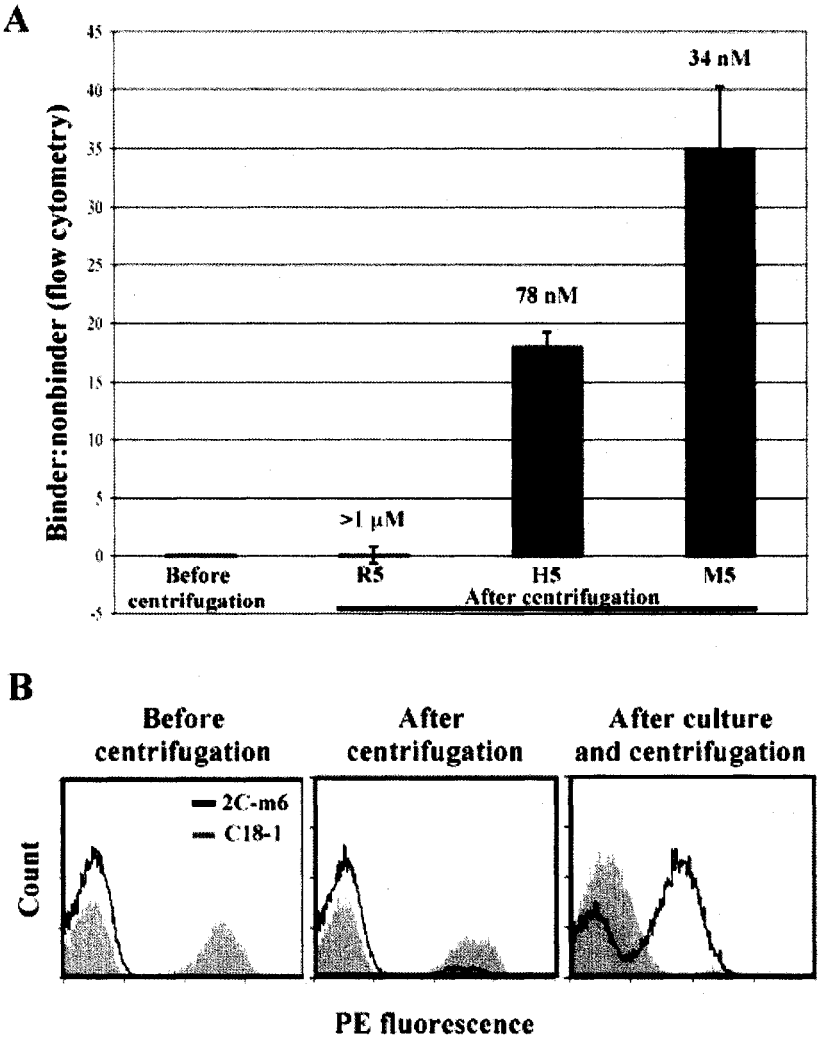

Figure 39: Selection efficiency across a range of affinities. A) T2-L^d cells were pulsed with the peptide variants R5, H5, and M5 and incubated with a yeast cell mixture comprising a 1 to 1000 ratio of the 2C-m6 to C18-1 TCRs. Aliquots of cells before and after centrifugation were cultured and stained with antibodies specific for the Vβ region of either C18-1 (KT-8C1) or 2C-m6 (F23.2) and analyzed using flow cytometry. The ratios of yeast cells staining positive for F23.2 (binder) to yeast cells staining positive for KT-8C1 (nonbinder) are shown before and after centrifugation (K_D value for each 2C-m6/peptide/L^d interaction is shown above bars). B) Flow cytometry histogram overlays showing C18-1-positive yeast (grey) and 2C-m6-positive yeast (black outline) before and after centrifugation following incubation with R5-loaded T2-L^d cells. The far right panel shows results following a second complete cycle of growth, induction, incubation with R5-loaded T2-L^d and centrifugation.

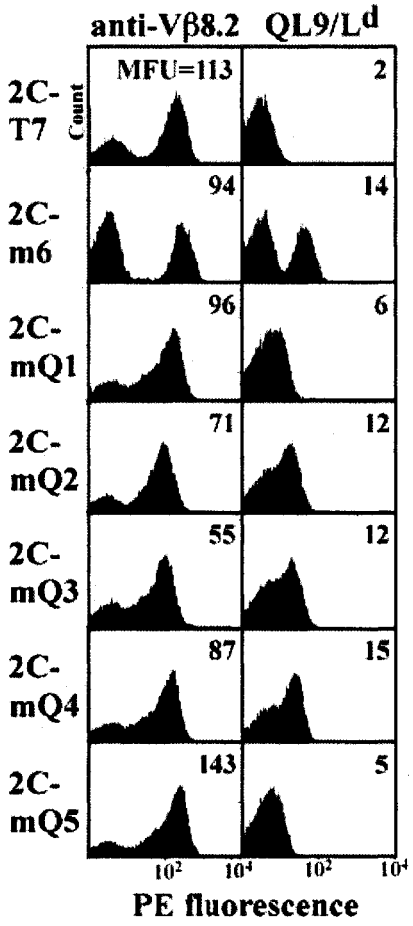

Figure 40: Analysis of unique 2C TCR mutants using flow cytometry. A) Histograms of yeast cells expressing mutant scTCRs 2C-T7, 2C-m6, and 2C-mQ-1 through 2C-mQ-5 incubated with either anti-Vb8.2 (F23.2) or QL9/L^d-Ig peptide-MHC dimers and PE-conjugated goat F(ab')2 anti-mouse Ig.

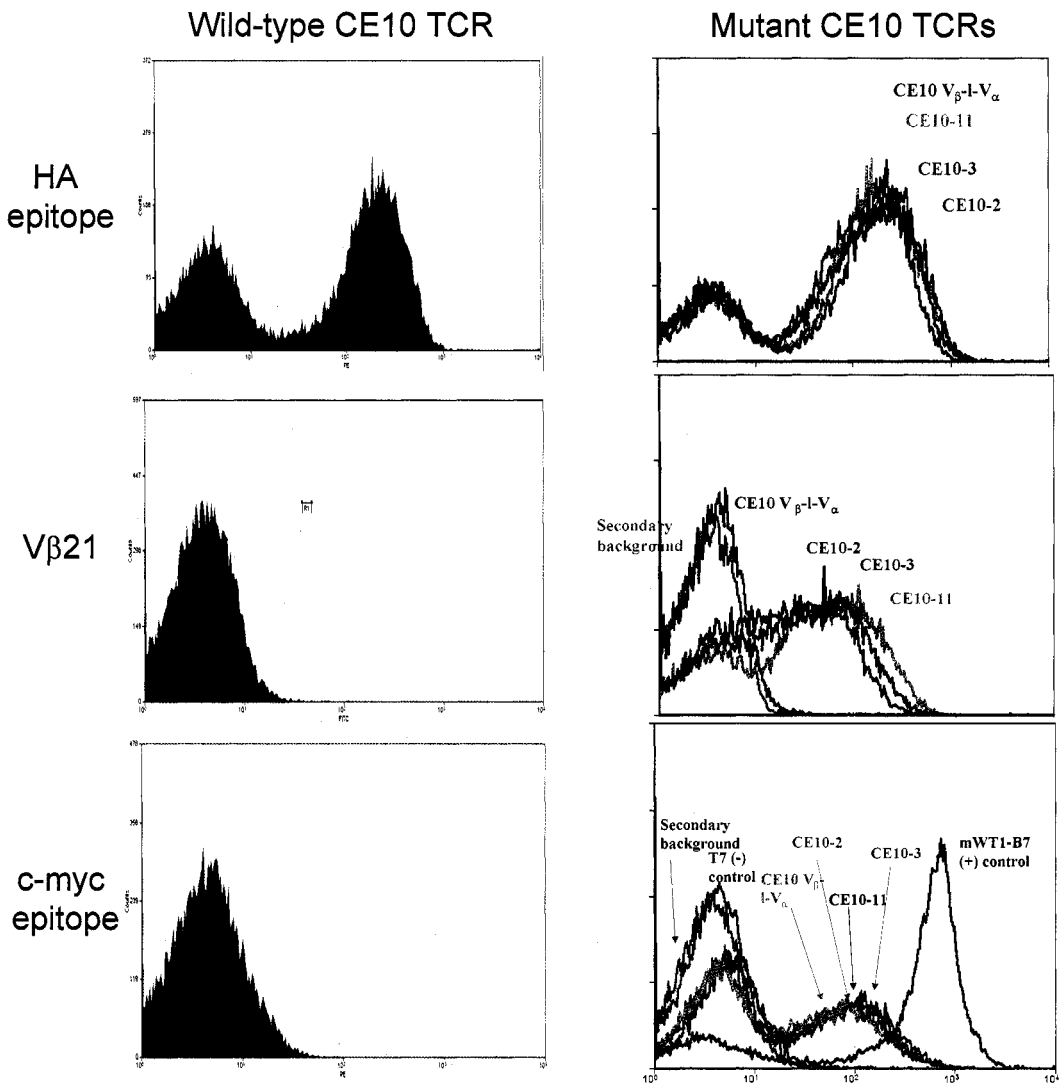

Figure 41: Mutations within the CE10-derived TCR improving yeast surface expression. Yeast expressing scTCRs of the original, wild-type Vα21Vβ21 TCR (CE10 Vβ-linker-Vα) and three mutants with improved surface expression (CE10-2, CE10-3, CE10-11). In the bottom right panel c-myc expression for the mWT1-B7 murine TCR mutant is shown for comparison. In a homogenous population of yeast expressing a single TCR, a negative staining population is always seen and serves as an internal control.

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