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Relationship Between CD8-Dependent Antigen Recognition, T Cell Functional Avidity, and Tumor Cell Recognition

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Abstract

Effective immunotherapy using T cell receptor (TCR) gene-modified T cells requires an understanding of the relationship between TCR affinity and functional avidity of T cells. In this study, we evaluate the relative affinity of two TCRs isolated from HLA-A2-restricted, gp100-reactive T cell clones with extremely high functional avidity. Furthermore, one of these T cell clones, was CD4⁺CD8⁺ indicating that antigen recognition by this clone was CD8 independent. However, when these TCRs were expressed in CD8⁺ Jurkat cells, the resulting Jurkat cells recognized gp100:209-217 peptide loaded T2 cells and had high functional avidity, but could not recognize HLA-A2⁺ melanoma cells expressing gp100. Tumor cell recognition by Jurkat cells expressing these TCRs could not be induced by exogenously loading the tumor cells with the native gp100:209-217 peptide. These results indicate that functional avidity of a T cell does not necessarily correlate with TCR affinity and CD8-independent antigen recognition by a T cell does not always mean its TCR will transfer CD8-independence to other effector cells. The implications of these findings are that T cells can modulate their functional avidity independent of the affinity of their TCRs.

Keywords

T cells; TCR; Melanoma; Affinity

Introduction

The ability of a vaccine to protect and/or treat an individual from infections or malignancies is dependent upon the quality and quantity of the immune response in the vaccinated host. In the case of cancer vaccines, the quality of the anti-tumor immune response is particularly critical given that most T cell antigens targeted on tumor cells are normal self proteins [1,2]. Immunologic tolerance to these self antigens may limit the T cell repertoire which in turn would limit the host's ability to mount an effective anti-tumor immune response. Furthermore, several mechanisms have been described in cancer patients such as downregulation of TCR ζ chain or

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the p56^{lck} kinase which can further impact on the quality of the anti-tumor immune response by impairing T cell function [3,4]. For cancer vaccines to succeed, it is critical that we understand what properties of a T cell immune response are required for the anti-tumor immune response to be most effective.

One critical measure of the quality of a T cell response to its antigen is avidity. High T cell avidity is generally accepted as a measure of the capacity of a T cell to recognize low levels of antigen as measured by cytokine release or cytolytic activity [5,6]. Given this measure of T cell function, it has been shown that high avidity correlates with the ability of a T cell to recognize tumor cells [5-7] and the therapeutic efficacy of that T cell [5,6]. Based on these observations, the goal of immunotherapy is to develop cancer vaccines that would elicit a high avidity T cell response against the tumor.

We have developed an alternative strategy for providing a patient with a source of autologous tumor reactive T cells. Using retroviral vectors, we were the first group to use TCR genes to successfully engineer normal peripheral blood T cells to recognize tumor cells [8]. Despite our ability to engineer tumor-reactive human T cells, it remains unresolved what properties of T cells predict whether their TCRs are suitable for engineering T cells with the greatest potential for anti-tumor efficacy. In fact, the two clinical trials where TCR gene modified T cell were used to treat patients had relatively few objective clinical responses [9,10]. Both of these trials used low affinity TCRs to engineer the T cells. One possibility is that TCRs with higher affinity would result in better T cells for adoptive transfer leading to improved clinical outcomes.

We have previously shown that CD8-independent tumor cell recognition may be a critical factor for identifying high-affinity tumor-responsive TCRs [11-13]. However, T cells capable of CD8 independent tumor cell recognition are quite rare and will be difficult to isolate for most tumors. Alternatively, strategies have been devised to isolate and expand T cells with the highest functional avidity for their antigen since T cell avidity has been shown to correlate with tumor cell recognition *in vitro* and tumor regression *in vivo* [5,6]. Therefore, we speculated that high avidity T cells would express TCRs with high affinity for their antigen.

In this study, we have cloned two TCRs reactive against the same HLA-A2/gp100:209-217 complex and characterized these TCRs for their relative ability to mediate tumor cell recognition. Our results indicate that the relative strength of a TCR cannot always be predicted from the biologic activity of the parent T cell. T cells appear to have other means of attenuating their functional avidity independent of the affinity of their TCR. Therefore, until further criteria can be shown to predict the affinity of a TCR from the function of a T cell, each TCR must be cloned and carefully evaluated in order to determine its ability to transduce the signals required for T cell activation.

Materials and Methods

Tumor cell lines and culture conditions

Unless otherwise indicated, all cell lines were grown in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS, from Invitrogen, Carlsbad, CA), 2mM L-glutamine (Invitrogen), 100 I.U./ml penicillin (Invitrogen), and 100 mg/ml streptomycin (Invitrogen). Melanoma (MEL) and renal cell carcinoma (RCC) cell lines, with the exception of MEL SK-23, were kindly donated by carcinoma patients undergoing surgical resection at the Surgery Branch, National Cancer Institute. HLA-A2-positive tumor lines included MEL 624, MEL 1300, MEL 1383, MEL SK-23, and RCC UOK 131. HLA-A2 negative tumor cell lines included RCC 1764, MEL 586 and MEL 624-28. Jurkat is a CD4⁺/CD8⁻ T-cell lymphoma that expresses an endogenous TCR. The T2 line is a TAP-deficient T-cell/B-cell hybridoma expressing HLA-A2. 293GP is a human renal cell line transfected to express the gag and pol retroviral genes,

which is grown in DMEM supplemented with 10% FBS, 2mM L-glutamine (Invitrogen), 100 I.U./ml penicillin (Invitrogen), and 100 mg/ml streptomycin (Invitrogen).

T Lymphocytes and TIL lines

Melanoma-reactive T cell clones, R6C12 and T4H2, were isolated from the peripheral blood of two separate melanoma patients that had received the gp100:209-217 210M peptide vaccine in incomplete Freund's adjuvant as part of a clinical trial in the Surgery Branch, NCI. The T cell clones were maintained in AIM-V media (Invitrogen) supplemented with 10% human AB serum (Valley Biomedical, Winchester, VA), 2mM L-glutamine, 100 I.U./ml penicillin, 100 mg/ml streptomycin, and 300 I.U. rhIL-2 (Chiron Corporation, Emeryville, CA) per ml. Melanoma-reactive tumor infiltrating lymphocyte (TIL) lines, TIL 1383I, TIL 1520, and TIL 1235 were established from surgical specimens obtained from melanoma patients as described [14]. TIL lines were maintained in AIM-V medium made as above except with 6000 I.U. rhIL-2 per ml. All of the T cells used in this study are HLA-A2 restricted and their reactivity is as follows: R6C12, T4H2 and TIL 1520 are reactive to the gp100:209-217 epitope, TIL 1383I is reactive to the tyrosinase:368-376 epitope, and TIL 1235 is reactive to the MART:27-35 epitope.

TCR identification

The identification of the TCR α and β genes from R6C12 and T4H2 were performed using a combination of 5' RACE followed by DNA sequencing and RT-PCR analysis using TCR AV and BV subfamily specific primers as described [15,16]. The identification, cloning, and expression of the TIL 5 and TIL 1383I TCRs have been described elsewhere [8,11,16]. 5' RACE was performed when it was determined that the presence of feeder cells (irradiated allogeneic PBMC) interfered with RT-PCR analysis using the TCR α and β subfamily specific primers. Using the protocol in the 5' RACE kit (Invitrogen, Grand Island, NY), first strand cDNA was synthesized from 1 μ g of total RNA, treated with RNase H and purified over a GlassMax column. One tenth of the purified cDNA was dC tailed using terminal deoxynucleotide transferase. The PCR reactions were performed in a 50 μ l final reaction volume using the equivalent of 25 ng of tailed cDNA, 4 pmoles of Anchor Primer, 2 pmoles of either TCR C α (CCTCAGCTGGACCACAGC) or C β (GGCAGACAGGACCCCTTG) specific primers, 2.5 units of Taq DNA polymerase (Continental Lab Products, San Diego, CA), and 200mM dNTP (Invitrogen). Amplification was performed for 35 cycles at 92° for 60 seconds, 54° for 60 seconds and 72° for 120 seconds followed by a 15 minute extension period at 72°. The products of each PCR reaction were ligated into the pCR 2.1 TA cloning vector (Invitrogen) and transformed into *E. coli* DH5 α competent cells (Invitrogen). Bacterial clones were screened by PCR using the specific TCR α and β primers to identify clones containing inserts of the predicted size. DNA was isolated from those clones and their inserts were sequenced by cycle sequencing using BigDye Terminator Cycle Sequencing kits (Applied Biosystems, Foster City CA) and analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The resulting sequences were analyzed using the Genetics Computer Group, Inc. software package [17]. TCR identification using TCR AV and BV subfamily specific primers: Total cellular RNA was isolated from 5×10^6 T cells using Trizol (Invitrogen) and cDNA was synthesized using oligo dT (12-18) (Invitrogen) and Superscript II reverse transcriptase (Invitrogen) as described [16]. The sequence of each TCR AV and BV subfamily specific primer has been described elsewhere [15]. Each TCR AV or BV subfamily specific forward primer was used in conjunction with its corresponding AC or BC reverse primer to amplify the TCR V genes used by the T cell clone. PCR reactions consisted of 200mM dNTP (Invitrogen), 400 nM of both forward and reverse primers, and 1 unit of Taq polymerase (Continental Lab Products) in a 50 :1 reaction. Amplifications were under the following conditions: 35 cycles of 92°C denaturation for 30 seconds, 60°C annealing for 30 seconds, and 72°C extension for 1 minute. PCR products were separated using ethidium bromide stained

agarose gels and electronically imaged under UV light. The presence of a band indicated transcripts using that TCR AV or BV subfamily were present in the T cell clone.

Plasmid and Retroviral Vectors

The SAMEN CMV/SR α retroviral vector has been described in detail elsewhere [8]. Key features of this retroviral vector are a hybrid 5' LTR containing the cytomegalovirus enhancer fused to the Moloney Murine leukemia virus LTR for increased retroviral titer, a second SR α promoter to permit expression of multiple transgenes, an IRES/neo cassette for drug selection, and unique restriction sites to facilitate subcloning of TCR chains.

Production of Retroviral Supernatants

Retroviral supernatants were produced by transiently cotransfecting 293GP cells with the retroviral vector and a plasmid encoding the VSV envelope gene as described [11]. After 24 hours, the medium discarded and replaced with fresh media. At 48 hours, 72 hours, and 96 hours, retroviral supernatants were harvested and either used immediately or frozen at -70°C.

Transfections and Transductions

Lipofectamine and Plus reagents (Invitrogen) were used for all transfections. To transduce cells, 500,000 Jurkat cells were suspended in 1 ml supernatant with 8 μ g polybrene in 24 well plates and centrifuged for 90 minutes at 1000 RCF. After transduction, cells were put into selection in complete media with 2 mg/ml G-418. After two weeks, the cells were cloned in limiting dilution and each clone was tested using cytokine release assays for human IL-2 (hIL-2) production when stimulated with peptide loaded T2 cells as described below. Clones with the highest reactivity were used in all subsequent experiments.

Cytokine release assays

For measuring hIL-2 release, 50,000 responder and 50,000 (sometimes peptide-loaded) stimulator cells were suspended per well of a 96 well plate in 200 μ l of growth media supplemented with 10 ng/ml PMA. The cells were left in the incubator at 37 °C overnight, the supernatants were harvested, and tested by ELISA or frozen at -70 °C for later testing.

For the human interferon-gamma (hIFN- γ) release, 5000 responder and 10,000 stimulator cells were suspended in 200 μ l per well in 96 well plates and let sit at 37 °C overnight. The supernatants were harvested the next day and either tested by ELISA or saved at -70 °C for later testing.

Immunofluorescence and Flow cytometry

Cell surface expression of T cell markers was measured by immunofluorescence staining and flow cytometry. All FITC and PE labeled monoclonal antibodies were purchased from BD Biosciences (San Jose, CA) 1×10^6 cells were stained with anti-CD3 FITC, anti-CD4 FITC, anti-CD8 FITC, anti-TCR $\alpha\beta$ PE, anti-TCR V β 12 PE, or anti-TCR V β 17 PE. Isotype matched FITC or PE labeled antibodies were used as controls for background staining. Relative log fluorescence of 10^4 live cells was measured using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Results

Reactivity and Phenotype of CTL Clones R6C12 and T4H2

We have previously reported the cloning and characterization of a melanoma reactive TCR that transfers CD8 independent tumor recognition to primary human T cells [12] and T cell lymphomas [11,12]. The analysis of this TCR and others that exhibit similar properties strongly

suggested that TCRs with high affinity for the antigen would be better suited for TCR gene transfer than those with lower affinity. The critical question that remains is how to identify such high-affinity TCRs. It has been suggested that T cell clones with high functional avidity express high affinity TCRs [5,6,18-22]. In order to test the relationship between T cell avidity and relative TCR affinity, we selected two gp100:209-217 reactive T cell clones, R6C12 and T4H2 for TCR cloning and characterization. Both R6C12 and T4H2 were HLA-A2 restricted (Figure 1A), secreted significant amounts (at least twice background and greater than 100 pg/ml) of interferon- γ when stimulated with T2 cells loaded with as little as 100 pg/ml of gp100:209-217 peptide (Figure 1B), and recognize HLA-A2⁺ gp100⁺ melanoma cells in interferon- γ release assays (Figure 1A). Based on their functional parameters, both T cell clones are considered to have high functional avidity.

Phenotypically, R6C12 is a conventional CD3⁺, CD4⁻, CD8⁺, TCR $\alpha\beta$ ⁺ T cell (Figure 2). Its extremely high functional avidity is consistent with the hypothesis that R6C12 would express a high affinity TCR. In contrast, T4H2 is an unconventional CD3⁺, CD4⁻, CD8⁻, TCR $\alpha\beta$ ⁺ T cell (Figure 2). Based on our previous experience with the TIL 1383I TCR which was isolated from an MHC class I restricted CD4⁺ CD8⁻ T cell, we hypothesized that the T4H2 TCR would mediate CD8-independent recognition of melanoma cells and would have high affinity for its ligand.

TCR Identification

As part of the molecular monitoring studies to determine the fate of R6C12 cells following adoptive transfer, we determined that R6C12 had a single β chain transcript using BV8 [23]. Expression of BV8 by R6C12 was confirmed by immunofluorescence using anti-V β 8 mAb (Figure 2). However, R6C12 expressed two α chain transcripts using AV12 or AV41. Since there are no mAb available that stain these TCR α chains, we relied on DNA sequence analysis to determine which TCR α chain was likely to pair with the BV8 chain to mediate antigen recognition. Based upon the DNA sequence, one α chain transcript used AV12S3/AJ36/AC and the other used AV41S1/AJ54/AC (data not shown). The AV12S3/AJ36/AC transcript encodes a TCR α chain protein which was “out of frame”. While the segment encoding the AV12S3 gene is correct, translation of the region encoding the AJ36 segment lacks the characteristic FGXG motif found in all TCR and Ig J segments [24] and the AC region is not in the correct reading frame, leading to an in-frame stop codon resulting in premature termination of the protein. In contrast, the AV41S1/AJ54/AC transcript encoded a productively rearranged protein that contained all of the necessary motifs characteristic of a functional TCR α chain. Therefore, we concluded that R6C12 used a TCR consisting of AV41S1/AJ54/AC and BV8S1/BD2S1/BJ2S1/BC2.

The TCR α and β chain genes used by T4H2 were identified by RT-PCR using AV and BV subfamily specific PCR primers. PCR analysis indicated that T4H2 had a single α and a single β chain transcript using AV12 and BV17 respectively (data not shown). The identification of the β chain was confirmed by immunofluorescence using anti-V β 17 mAb (Figure 2). Again, no mAbs were available that stain the TCR α chains. Based on the DNA sequence, we determined that the AV12S2/AJ49/AC transcript expressed by T4H2 encoded a productively rearranged protein that contained all of the necessary motifs that are characteristic of a productively rearranged TCR α chain. Therefore, we concluded that T4H2 used a TCR consisting of AV12S2/AJ49/AC and BV8S1/BD2S/BJ2S7/BC2.

Expression and Function of R6C12 and T4H2 TCR in Jurkat Cells

Full length cDNAs encoding the R6C12 and T4H2 α and β chains were amplified by RT-PCR using specially designed cloning primers to permit rapid subcloning into the SAMEN CMV/SR α backbone. PCR products were then ligated into the pCR 2.1 TA cloning vector, and the

inserts were sequenced to ensure no PCR errors were introduced. The R6C12 or T4H2 α and β chains were subcloned into the SAMEN CMV/SR α retroviral vector with the α chains being expressed by the promoter in the LTR and the β chain being expressed by the internal SR α promoter [8]. Retroviral supernatants were produced by transiently co-transfecting 293GP packaging cells with the retroviral construct and a plasmid encoding the VSV envelope gene. Jurkat cells were then transduced with R6C12 and T4H2 TCR retroviral supernatants. As controls, Jurkat cells were transduced with a low affinity TCR (TIL 5) or a high affinity TCR (TIL1383I). As shown in Table 1, each TCR-transduced Jurkat culture secreted IL-2 when stimulated with T2 cells loaded with their corresponding antigen but did not respond to tumor cells carrying the same antigen. However, the TIL 1383I Jurkat cells secreted IL-2 when stimulated with HLA-A2⁺ melanoma cells (Table 1). These results were somewhat surprising since the extremely high avidity of R6C12 (Figure 1B) and the CD8 independent tumor recognition by T4H2 (Figures 1A and 2) predicted they both expressed high affinity TCRs capable of transferring tumor-specificity.

It has previously been reported that a T cell's ability to recognize tumor cells correlates with its functional avidity [7]. Therefore, it is possible that the R6C12 and T4H2 TCR-transduced Jurkat cells failed to recognize tumor cells because they have low functional avidity. This is not the case since both R6C12 and T4H2 Jurkat cells secreted significant amounts of IL-2 when stimulated with T2 cells loaded 100 pg/ml of gp100:209-217 peptide (Figure 3). As a further means of assessing the function of the Jurkat cells expressing the R6C12 and T4H2 TCRs, we stimulated them with peptide loaded tumor cells. As shown in Table 2, TIL 1383I Jurkat, TIL 1520, and TIL 1235 cells could recognize the HLA-A2⁺ melanoma cells (624 MEL and SK23 MEL) but not HLA-A2⁺ renal cell carcinoma cells (RCC 131). As before, R6C12 and T4H2 Jurkat cells failed to recognize the HLA-A2⁺ melanoma cells. We had previously shown that the low affinity TIL 5 TCR could recognize HLA-A2⁺ melanoma cells if they were pre-loaded with the MART-1:27-35 peptide [25]. However, neither the R6C12 nor the T4H2 Jurkat cells were able to secrete IL-2 when stimulated with tumor cells loaded with gp100:209-217 peptide. These tumors presented the exogenously loaded gp100 peptide to T cells since the HLA-A2⁺ renal cell carcinoma cells stimulated TIL 1520 to secrete interferon- γ . We then speculated that there was insufficient gp100 antigen present on the peptide loaded cells to stimulate the R6C12 and T4H2 Jurkat cells. To circumvent this concern, we loaded the tumor cells with the modified gp100:209-217 210M peptide. Substituting a M for the T at position 210 in the gp100:209-217 peptide enhances binding of the peptide to HLA-A2 by 9-fold relative to the unmodified peptide [26]. By enhancing peptide binding to the MHC, MHC surface expression may be stabilized and enhanced [27-29]. Increasing the amount of peptide bound to HLA-A2 did allow the T4H2 Jurkat cells to recognize the melanoma and renal cell tumors (Table 2). However, these gp100:209-217 210M peptide loaded tumors were still unable to stimulate R6C12 Jurkat cells to secrete IL-2. These results suggested that the R6C12 and T4H2 TCRs had lower affinity for antigen than our low affinity TIL 5 TCR.

Discussion

Understanding how the affinity of a TCR relates to the ability of its T cell to respond to antigen is extremely important for rationally choosing the best candidate T cells to clone TCRs for use in engineering T cells for adoptive immunotherapy. It has been suggested that T cells with high functional avidity express high affinity TCRs [5,6,18-22]. If true, we would be able to predict the relative affinity of a TCR based on functional parameters of the parent T cell and it would enable us to rapidly screen for T cells bearing high affinity TCRs. However, this assertion has never been directly tested by cloning and characterizing the TCRs from T cell clones with high functional avidity.

In this study, we tested this hypothesis using two T cell clones that have extremely high functional avidity and can recognize tumor cells. Surprisingly, when the TCRs from the T4H2 and R6C12 T cell clones were transferred to Jurkat cells, they were unable to recognize the physiologic levels of antigen expressed by tumor cells. We have shown that TIL 5 TCR transduced Jurkat cells which can't recognize HLA-A2⁺ melanoma cells, can recognize these tumors when they are loaded exogenously with the MART-1:27-35 peptide [25]. We concluded that weak signaling by the low affinity TIL 5 TCR can be overcome by increasing the amount of antigen presented by the tumor cell [25]. This was not the case with the R6C12 and T4H2 TCRs. T4H2 Jurkat cells can only recognize tumor cells loaded with the gp100:209-217 210M peptide which has higher affinity for HLA-A2 than the wild type peptide. R6C12 Jurkat cells are unable to recognize any of the tumor targets, even when they are loaded with the gp100:209-217 210M peptide. Based on this analysis, it is reasonable to conclude that both the R6C12 and T4H2 TCRs have lower affinity than the TIL 5 TCR with the R6C12 TCR being the weakest of the three.

There are several conclusions from this study. First is that the functional avidity of a T cell does not always correlate with the affinity of its TCR. In addition to the results presented here, we have further evidence to support this conclusion. We have shown that CD4⁺ TIL 1383I cells and any T cell expressing the TIL 1383I TCR can recognize melanoma cells in the absence of CD8 expression [11,12,30]. In peptide titration experiments, it takes T2 cells loaded with 10-100 ng/ml of peptide to stimulate cytokine secretion from TIL 1383I T cells [30], and TIL 1383I TCR expressing 58 $\alpha\beta^+$ cells [11], Jurkat cells [12], or normal PBL-derived T cells [12]. Therefore, TIL 1383I cells represent the opposite situation where T cells with low functional avidity express a high affinity TCR.

A second conclusion from this study is that a TCR cloned from a T cell that exhibits CD8 independent tumor cell recognition does not always have adequate affinity to transfer CD8 independence to other T cells. Our studies with the TIL 1383I TCR were the first to suggest that T cells exhibiting CD8 independent tumor cell recognition expressed high affinity TCRs [11,12]. Theobald et al [31] reported that T cells isolated from HLA-A2/K^b transgenic mice recognize p53⁺ human tumors. Since mouse CD8 does not bind to the $\alpha 3$ domain of human HLA, any mouse T cell that recognizes human tumors would be defined as CD8 independent. When the TCR from a T cell clone isolated from a p53 reactive T cell culture was expressed in CD8⁻ T cells, this mouse p53 TCR indeed transferred CD8 independent tumor cell recognition [32]. This was a particularly important observation because it suggested that mouse T cells capable of recognizing antigens in the context of human HLA class I molecules would express high affinity TCRs which could be useful for patient treatment. However, a recent study by Santomaso et al [33] reported contradictory results when they cloned a TCR from a cdr2 reactive T cell clone isolated from an HLA-A2/D^d transgenic mouse. Despite the parent clone exhibiting CD8 independent recognition of human tumors, the authors reported no evidence of TCR gene modified T cells being CD8 independent. Therefore, our results support the notion that a T cell clone that has CD8 independent tumor cell recognition does not necessarily express a high affinity TCR.

The key question that remains unanswered is why do these T cells have such high functional avidity yet express low affinity TCRs? The likely explanation is that a T cell can "attenuate" its functional avidity independent of the strength of its TCR (34). A classic example of this phenomenon is peripheral anergy. The Schwartz group demonstrated that T cell clones can be made antigen unresponsive [35] and that IL-2 can reverse this antigen unresponsiveness [36]. The ability of IL-2 to reverse T cell anergy is further supported by the mouse and human TIL studies reported by the Rosenberg group [14,37]. In these studies, T cells that are refractory to antigen stimulation in a tumor lesion can be activated *ex vivo* by IL-2 leading to fully functional T cell cultures that can mediate tumor regression *in vivo* [37,38]. The Alexander-Miller group

has studied the mechanism of modulating T cell reactivity as measured by functional avidity using CD8⁺ T cells from TCR transgenic T cells. It is clear from their studies that CD8 plays a critical role in regulating the functional avidity of a T cell [39-41]. Their conclusions were that a T cell clone has the potential to differentiate into cells with high or low functional avidity [40,41]. We have examined what properties of CD8 can impact T cell function using Jurkat cells expressing high and low affinity human TCRs [42]. In our hands, enhancing the strength of the TCR/peptide/MHC interaction using modified CD8 chains does not necessarily improve T cell function. For TCRs with high affinity, the signaling function of CD8 which translocates the kinase lck to the CD3 complex appears to be more important for enhancing T cell function than the role of CD8 in stabilizing the TCR/peptide/MHC interaction. For TCRs with lower affinity, both the structural and signaling roles of CD8 are important.

Another important question worth consideration is how TCR structure impacts on the function of a T cell. We have previously reported isolating a TCR from an HPV E7 reactive T cell clone that fails to signal when transferred to other T cells (43). The identification of the TCR was correct because TCR transduced cells specifically stained with HPV E7:11-20 peptide loaded HLA-A2 tetramers. It has been reported that TCRs as well as components of the CD3 complex undergo conformational changes upon antigen engagement (44-46). More importantly, sequences within the TCR α and β chains are known to impact on TCR expression, pairing, and function in T cells (47-51). Therefore, it might be reasonable to conclude that sequences in a cloned TCR can impact on its ability to transduce T cell activation signals and mediate proper CD3 assembly after encountering antigen. This may account for why the same TCR can function well in the parent T cell yet poorly when expressed by another T cell.

In summary, the work we report here further supports the notion that a T cell's ability to recognize antigen is independent of the strength of its TCR. We consider this to be good news for the development of immune-based therapies for cancer. Even though we cannot easily alter the genetics of our T cells, there are agents available such as IL-2, IL-12 and others capable of altering their physiology. Therefore, a better understanding of the mechanisms that regulate T cell function may lead to improved TCR-based immune therapies.

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Figure 1. Immune Reactivity of T Cell Clones R6C12 and T4H2

T cell clones R6C12 and T4H2 were selected for this study based on their ability to recognize tumor cells and their functional avidity. A) Tumor recognition by T cell clones R6C12 and T4H2 was assessed using interferon- γ release assays. HLA-A2⁺ gp100⁺ (624 MEL, 1300 MEL, 1383 MEL), HLA-A2⁻ gp100⁺ (624-28 MEL, 586 MEL), and HLA-A2⁻ gp100⁻ (RCC 131) cells were cocultured overnight with T cell clone R6C12 and T4H2 and the amount of interferon- γ released was measured by ELISA. As controls, the amount of interferon- γ released by TIL 1235 (anti-MART-1) and TIL 1383I (anti-tyrosinase) when cocultured with the same targets was measured. The amount of interferon- γ released was measured by ELISA. B) The functional avidity of T cell clones R6C12 and T4H2 was measured in interferon- γ release assays. T cells were stimulated with T2 cells loaded with decreasing concentrations of

gp100:209-217 peptide. The amount of interferon- γ released was measured by ELISA. Relative avidity was defined as the minimum amount of peptide loaded on T2 cells that could elicit significant cytokine release. Significant cytokine release was considered to be at least twice background and greater than 100 pg/ml. Each point represents the average of triplicate wells and is a representative of three replicate experiments. It should be noted that the data in panels A and B were from the same experiment. As a result, the specificity controls for panel B can be found in panel A.

Figure 2. Cell Surface Phenotype of T Cell Clones R6C12 and T4H2

The expression of T cell markers on T cell clones R6C12 and T4H2 was measured by immunofluorescence. Cells were stained with FITC conjugated anti-CD3, CD4, and anti-CD8 monoclonal antibodies or PE conjugated anti-TCR $\alpha\beta$, V β 8, and V β 17 monoclonal antibodies. The log fluorescence of 10^4 live cells was measured using a FACScan flow cytometer.

Figure 3. Functional Avidity of TCR transduced Jurkat cells

The relative sensitivity of R6C12 and T4H2 TCR transduced Jurkat cells was measured using IL-2 release assays using T2 cells incubated with different concentrations of gp100 209-217 peptide. The amount of IL-2 released was measured by ELISA. Relative avidity was defined as the minimum amount of peptide loaded on T2 cells that could elicit significant cytokine release. Significant cytokine release was considered to be at least twice background and greater than 100 pg/ml. Each point represents the average of triplicate wells and is a representative of three replicate experiments. It should be noted that the data in this figure and in Table 1 were from the same experiment with T2 cells loaded with 10 μg/ml gp100 209-217 peptide data

point being shown in both. As a result, the specificity controls for this figure can be found in Table 1.

Table 1

Antigen Recognition by Jurkat cells Expressing Melanoma Reactive TCRs. The reactivity R6C12 and T4H2 TCR transduced Jurkat cells was measured in TCR transduced Jurkat cells were stimulated with T2 cells alone or exogenously loaded with melanoma peptides and a panel of cytokine release assays. Peptides used in this experiment were the native gp100:209-217 peptide, the modified gp100:209-217 210M peptide, and control tyrosinase: 368-376 and MART-1:27-35 peptides. Tumor cells used in this experiment included HLA-A2⁺ and HLA-A2- melanoma cell lines (586 MEL, 624 MEL, 624-28 MEL, 1300 MEL, 13831 MEL) and an HLA-A2⁺renal cell carcinoma cell line (RCC 131). As controls for the responder cells, untransduced, TIL 5 TCR, and TIL 13831 TCR transduced Jurkat cells were included in the assay. The amount of IL-2 released was measured by ELISA. Each value represents the mean of triplicate wells and is representative of three replicate experiments. Values listed as <32 were below the lowest value on the standard curve. Underlined values represent significant IL-2 release, which is defined as at least twice background and greater than 100 pg/ml.

Stimulators	HLA-A2 Expression	Medium	Jurkat	Responder Cells		
				TIL 1383I Jurkat	TIL 5 Jurkat	R6C12 Jurkat
Medium	N/A	<32	<32	<32	<32	<32
T2 alone	-	<32	<32	<32	<32	<32
T2 + MART-1:27-35	+	<32	<32	<32	<u>1,025</u>	<32
T2 + tyrosinase:368-376	+	<32	<32	<u>1,219</u>	<32	<32
T2 + gp100:209-217	+	<32	<32	<32	<32	<32
T2 + gp100:209-217 210M	+	<32	<32	<32	<u>1,051</u>	<u>4,033</u>
624-28 MEL	-	<32	<32	<32	<u>677</u>	<u>10,033</u>
624 MEL	+	<32	<32	<32	<32	<32
586 MEL	-	<32	<32	430	<32	<32
1300 MEL	+	<32	<32	<32	<32	<32
1383 MEL	+	<32	<32	<u>253</u>	<32	<32
RCC 131	+	<32	<32	<u>258</u>	<32	<32
	+	<32	<32	<32	<32	<32

Table 2

Antigen Recognition by Jurkat cells Expressing Melanoma Reactive TCRs. The reactivity R6C12 and T4H2 TCR transduced Jurkat cells was measured in cytokine release assays. TCR transduced Jurkat cells were stimulated with T2 cells or HLA-A2⁺ tumor cells alone or exogenously loaded with melanoma peptides. Peptides used were the native gp100:209-217 peptide, the modified gp100:209-217 210M peptide, or control tyrosinase:368-376 peptide. As controls for the responder cells, untransduced and TIL 1383I TCR transduced Jurkat cells were included in the assay. As controls for the stimulators, TIL 1520 and TIL 1383I were included in the assay. The amount of cytokine released (IL-2 for Jurkat cells and interferon- γ for TIL cells) was measured by ELISA. Each value represents the mean of triplicate wells and is representative of three replicate experiments. Values listed as <32 were below the lowest value on the standard curve. Values listed as >2,000 exceeded the highest value on the standard curve. Underlined values represent significant IL-2 release, which is defined as at least twice background and greater than 100 pg/ml.

Stimulators	Jurkat	R6C12 Jurkat	T4H2 Jurkat	Responder Cells	
				TIL 1383I Jurkat	TIL 1383I
T2 alone	<32	<32	<32	<32	<32
T2 + tyrosinase:368-376	<32	<32	<32	>2,000	>2,000
T2 + gp100:209-217	<32	459	1,189	<32	<32
T2 + gp100:209-217 210M	<32	<u>363</u>	<u>>2,000</u>	<32	<32
RCC I31	<32	<32	<32	<32	<32
RCC I31 + gp100:209-217	<32	<32	<32	<32	<32
RCC I31 + gp100:209-217 210M	<32	<32	894	<32	<32
624 MEL	<32	<32	<32	<u>167</u>	940
624 MEL + gp100:209-217	<32	<32	<32	<u>182</u>	956
624 MEL + gp100:209-217 210M	<32	<32	<u>1,857</u>	<u>185</u>	958
SK23 MEL	<32	<32	<32	<u>151</u>	>2,000
SK23 MEL + gp100:209-217	<32	<32	<32	<u>159</u>	>2,000
SK23 MEL + gp100:209-217 210M	<32	<32	<u>1,455</u>	<u>131</u>	>2,000