Adaptive T-cell therapies have shown significant promise in the treatment of cancer and viral diseases. One approach, which introduces antigen-specific T-cell receptors (TCRs) into ex vivo activated T cells, is designed to overcome central tolerance mechanisms that prevent responses by endogenous T-cell repertoires. Studies have suggested that use of higher-affinity TCRs against class I major histocompatibility complex antigens could drive the activity of both CD4⁺ and CD8⁺ T cells, but the rules that govern the TCR binding optimal for in vivo activity are unknown. Here, we describe a high-throughput platform of ‘reverse biochemistry’ whereby a library of TCRs with a wide range of binding properties to the same antigen is introduced into T cells and adoptively transferred into mice with antigen-positive tumors. Extraction of RNA from tumor-infiltrating lymphocytes (TILs) or lymphoid organs allowed high-throughput sequencing to determine which TCRs were selected in vivo. The results showed that CD8⁺ T cells expressing the highest-affinity TCR variants were deleted in both the TIL population and in peripheral lymphoid tissues. In contrast, these same high-affinity TCR variants were preferentially expressed within CD4⁺ T cells in the tumor, suggesting they had a role in antigen-specific tumor control. The findings thus revealed that the affinity of the transduced TCRs controlled the survival and tumor infiltration of the transferred T cells. Accordingly, the TCR library strategy enables rapid assessment of TCR-binding properties that promote peripheral T-cell survival and tumor elimination.

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**INTRODUCTION**

The interaction between T-cell receptor (TCR) and peptide/major histocompatibility complex (MHC) is the key recognition event initiating functional T-cell responses. While much has been learned about the structure and function of this interaction,1–4 many aspects of the processes that operate in vivo remain unknown. This is particularly relevant in T-cell-based cancer therapies, where the questions include what TCR-binding affinities will achieve optimal destruction of tumors, drive differentiation into distinct effector types and promote persistence of peripheral T cells.

While there are many examples of tumor-infiltrating lymphocytes (TILs) that have been isolated from human cancers,5 these T cells have rarely been cloned or their TCRs examined owing to lower surface expression levels of the exogenous TCR, and whether different affinities drive the activities and differentiation of the plethora of T-cell subsets.6–9 Recently, strategies have been developed to delineate the fate of T cells not only on a population-wide scale but also on a single-cell basis. These systems range from intravital imaging to retrovirally introduced unique DNA sequences (termed ‘cellular barcodes’), allowing the fate of single naive T cells to be tracked under different conditions.10,11 While progress has been made in elucidating the mechanisms involved in T-cell lineage decisions using these techniques, there remains a need to more rapidly assess the relationships between TCR-binding properties and T-cell function, persistence and lineage commitment in vivo. This information would be of significant value in the choice of TCRs for adoptive T-cell therapies.12–14

Prompted by this lack of a high-throughput strategy to explore optimal tumor-targeting properties of T cells with exogenous TCRs, here we describe a system based on the rational design of TCR libraries with a wide range of affinities for a model cancer antigen. To develop the system, we took advantage of the well-studied binding properties and structures of the 2C TCR,15 specific for class I MHC Kb bound to a foreign peptide SIYr,16 and its high-affinity TCR variant called m33.17,18 The generation of single amino-acid substitution libraries in 2C and m33 provided a 10 000-fold range in binding affinities among the TCR variants. The approach can be applied to virtually any TCR, given that mouse and human TCRs have several CDR1 or CDR2 residues that are energetically important in MHC binding.2,3

**Gene Therapy (2013) 20, 634–644; doi:10.1038/gt.2012.80; published online 11 October 2012**

**Keywords:** TCR; T-cell display; adoptive T-cell therapy; affinity engineering; tumor targeting
Here we defined, in one sorting step, TCR variants that bound with the highest affinities (the ‘binding signature’) to the antigen in vitro and compared them with what was selected when libraries containing these variants were introduced into CD4+ and CD8+ T cells and transferred into tumor-bearing mice. CD8+ T cells expressing the highest-affinity TCRs were deleted in vivo (in the tumor and lymphoid tissues), whereas the CD4+ T cells expressing these TCRs remained in the periphery, and were present at higher levels in the tumor. However, even CD4+ T cells with the lowest-affinity TCRs were capable of infiltration into the tumor. The TCR library approach provides a platform for understanding the many aspects of how TCR binding operates to control T-cell function in vivo.

RESULTS

Design principles for TCR libraries with diverse binding affinities

To overcome limitations with characterizing TCR-binding properties of TILs, we adopted an approach in which a library of related TCRs was engineered. The strategy took advantage of findings that a single residue in CDR2 of TCR variable regions contributes significant binding energy.2,3,19,20 We used the 2C TCR and its high-affinity variant m33 (with affinity-increasing mutations in CDR3a). These TCRs bind with known affinity to the foreign peptide SIYRYYGL (SIY) and the structurally similar self-peptide EQYKFYSV (dEV8), both restricted by Kb (Figure 1a).21–23 2C recognizes SIY/Kb as a strong agonist, while dEV8/Kb could potentially act in positive selection.24 Affinities of 2C and m33 for SIY/Kb are 1000-fold different (30 μM and 30 nM, respectively), yet their affinities for dEV8/Kb are only about twofold different (80 μM and 40 μM, respectively).17,25 Nevertheless, CD8+ T cells (but not CD4+ T cells) that express the m33 TCR are capable of being stimulated by dEV8,17 suggesting that dEV8/Kb on normal cells in an H-2b mouse could interact productively with m33 CD8+ T cells.

Two residues of the 2C CDR2β, Y46β and Y48β that dock over the α1 helix of Kb26 are energetically important for SIY/Kb binding (Figure 1b). As the m33 alanine mutant of Y46β exhibited a 100-fold reduction in affinity,18 we reasoned that a library of all possible 20 amino-acid substitutions in a single residue such as Y46β introduced into 2C and m33 would provide a wide range of affinities, over 10 000-fold (Figure 1c). It is possible to generate TCR libraries in retroviral vectors for transducing T cells.27,28 Thus, we applied this strategy to an in vivo system, with the potential to extract information directly from TILs and lymphoid tissue, by generating position 46β libraries (NNS) in the pMP71 vector29,30 that contained 2C and m33 TCRs with disulfide-stabilized constant regions.31–33 While the libraries used here were relatively small (two libraries of 21 TCR variants each, including the stop codon), this strategy represents a valid way to evaluate the method, as it covers a wide range of affinities for our target and includes many variants that have not been characterized and several variants for which we have significant knowledge of the boundaries and properties of pepMHC binding. In the future, the technique need not be

Figure 1. Design and characterization of 2C and m33 Y46β libraries. (a) Peptides recognized by the 2C and m33 TCRs in the context of Kb: the model tumor antigen SIYRYYGL (SIY) in blue and self-peptide EQYKFYSV (dEV8) in red. (b) Location of Y46β in the CDR2β of 2C and m33 relative to the α1 helix on Kb. (c) Affinity spectrum covering all Y46β library residues in 2C and m33 for the model tumor antigen SIY/Kb: m33-wt having the highest affinity (KD = 0.03 μM) and 2C-Y46β[A having the lowest affinity (KD > 300 μM), representing a 10 000-fold difference. (d) Residue frequency of Y46β library, expected (teal bars) and observed for 2C (blue bars) and m33 (red bars), using the NNS library, where ‘N’ is any base and ‘S’ is either cytosine or guanine giving a total number of 32 possible codons. The number of sequences analyzed by 454 sequencing for the 2C library is 901 and 10 604 for the m33 library.
restricted to a single-codon degeneracy: each mouse received \( \sim 7 \times 10^6 \) transduced T cells, and transduction efficiencies ranged from 50 to 80%; a library of about \( 10^6 \) T cells could cover the diversity of four degenerate codons (32').

In order to examine codon frequencies present in the libraries, DNA from pMP71/TCR-transformed *Escherichia coli* was subjected to PCR and 454 sequencing. The observed frequencies were consistent with expected (NNS) frequencies (Figure 1d), although several slightly over- or under-represented codons were present within each library. Nevertheless, all amino acids were represented in the libraries, and the *E. coli* frequencies could be used for comparison with various frequencies 'selected' in vitro and in vivo.

Strong amino-acid bias in the CDR2\(\beta\) libraries selected in vitro for binding by SIY-K\(^\beta\)

To examine the importance of Tyr 46\(\beta\) in pepMHC binding, selections in vitro were performed. First, 2C and the 2C library were transduced separately into a T-cell hybridoma that expresses CD8\(\alpha\)(cell surface binding of SIY/K\(^\beta\) multimers by 2C requires CD8).34,35 Based on staining of the 2C/CD8\(^+\) T cells (Figure 2a), 10 nM SIY/K\(^\beta\)-Ig dimers were used to sort the 2C library without interference by TCR-independent CD8 binding. After one round of sorting, RNA was isolated from the selected cells, and reverse transcriptase PCR/454 sequencing was performed (Figure 2b). While Tyr was a predominant residue, Phe and His also were highly selected. Comparison of the ratio of frequencies in the

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Selection of SIY/K\(^\beta\)-binding 'signature' residues in TCRs at position 46\(\beta\). (a) SIY/K\(^\beta\) binding to T-cell hybridoma cells transduced with 2C (left, top panel) or m33 (right, top panel) TCRs, with or without co-expression of CD8\(\alpha\), respectively. T-cell hybridomas expressed with (top panels) or without (bottom panels) the TCRs were stained with the indicated concentrations of SIY/K\(^\beta\)-Ig dimer, followed by PE-labeled goat anti-mouse Ig, and analyzed by flow cytometry. (b) Frequency of residues from SIY/K\(^\beta\)-selected TCR libraries. T-cell hybridomas were transduced with a library of TCRs with variation at position 46\(\beta\) in the 2C or m33 TCR, with or without co-expression of CD8\(\alpha\), respectively. The top 1.4% of SIY/K\(^\beta\)-Ig dimer-binding T cells were sorted, RNA was isolated and cDNA was used for PCR, followed by 454 sequencing. The frequencies measured for each amino-acid residue at position 46\(\beta\) are shown for the 2C TCR library (selected with 10 nM SIY/K\(^\beta\)-Ig dimer, left panel, representing 29,754 sequences) and m33 TCR library (selected with 2 nM SIY/K\(^\beta\)-Ig dimer, right panel, representing 18,560 sequences). (c) Relative representation of individual amino-acid residues from the original, pre-selection library were analyzed by plotting the logarithm of the frequency of each residue in the SIY/K\(^\beta\)-selected library divided by the frequency for that residue in the original *E. coli* library (Log(residue frequency, selected/residue frequency, original)). A value of +1 indicates the residue is 10-fold over-represented compared with the unselected library, while a value of −1 indicates the residue is 10-fold under-represented compared with the unselected library. Representative data are shown from four experiments. For the signature residues (Met, Phe, Trp, Tyr and His) selected from the 2C library, the Tyr, Phe and His residues were significantly enhanced (\(P < 0.001\)). For the signature residues selected from the m33 library, the Tyr, Phe, His and Trp residues were significantly enhanced, and the Met was significantly reduced (\(P < 0.001\)).
selected population vs the E. coli library revealed that five residues were selected for SIY/Kβ binding (called the ‘binding signature’ here); Phe = Tyr = His > Met = Trp (Figure 2c). Thus, although Tyr is a preferred residue at position 46 of the CDR2β, other side chains allow significant MHC binding as well.

A similar experiment was performed with m33 and the m33 library, both transduced into the T-cell hybridoma without CD8 (m33 TCR does not require CD8 for staining with SIY/Kβ-Ig dimers; Figure 2a). A concentration of 2 nM SIY/Kβ-Ig dimers was used for sorting, and for m33, Phe was again a predominant selected residue (Figure 2b); relative to E. coli frequencies, a ‘binding signature’ similar to 2C was observed (the only differences for m33 were that Trp appeared to be more prevalent, and Met less prevalent, than seen for 2C) (Figure 2c).

Antigen-bearing tumors are infiltrated by T cells transduced with libraries of high- and low-affinity TCRs

To apply the selection of TCR from libraries in vivo, we used SIY as a model tumor antigen.36,37 The parental melanoma line B16-F10 and the SIY-transfected line B16-SIY were used as transplantable, subcutaneous tumors for these studies.38 B16-F10 and B16-SIY tumor cells were transplanted bilaterally and 5 days later, when tumors were readily palpable, ~7 x 10^5 T cells of various transduced preparations were introduced (Supplementary Figures 1 and 2). As a source of T cells, we used purified CD4+ or CD8+ T-cell populations from C57BL/6 mice, activated in vitro with anti-CD3/anti-CD28-coated beads and interleukin-2 (IL-2). Based on previous kinetic analyses of T-cell infiltration into tumors,39,40 mice were killed 5 days after adoptive transfer for immunohistology and TCR sequence analyses.

**Table 1.** Immunohistology of tumors showed that 2C-transduced CD8^+ T cells (Figure 3a) and 2C-transduced CD4^+ T cells, but not the mock-transduced T cells, infiltrated the antigen-bearing tumors (Figure 3b). No significant infiltration was seen by any of the T-cell populations in the B16 parental tumors (data not shown). Notably, the m33-transduced CD8^+ T cells were not present in B16-SIY tumors, whereas m33-transduced CD4^+ T cells were present. The absence of m33-transduced CD8^+ T cells is consistent with our recent observation that these cells are deleted within hours of transfer into an H-2b mouse.33 In general, 2C- or m33-transduced CD4^+ cells tended to be present at lower levels than the 2C-transduced CD8^+ T cells. Both CD4^+ and CD8^+ T cells transduced with the 2C and m33 libraries exhibited extensive infiltration into the B16-SIY tumors. Accordingly, the m33 library of TCRs in CD8^+ T cells presumably had a substantial number of variants that did not result in deletion as observed for the Tyr46β-containing m33 (Figures 2b and c).

In order to assess whether some of the infiltrating T cells in the B16-SIY tumors might have been non-specific, and possibly present owing to antigen-specific (SIY) T-cell-mediated inflammation, we stained tumor sections from 2C-transduced T cells with the clonotypic antibody 1B2 and with anti-CD3 (Figure 3c). This staining revealed that of the CD3^+ cells, ~60% were 1B2-positive and 40% were 1B2-negative. Thus, although antigen-specific T cells were necessary to observe significant T-cell infiltration, many of the T cells present appeared to express only their endogenous TCRs and were presumably not antigen specific.

Over the 5-day period before analysis by histology and sequencing (below), we measured tumor sizes to assess immediate functional responses. In order to easily compare 17 different

![Figure 3](image_url)
treatment groups, the growth rates of the tumors (in mm³ per day) were determined from measurements over the 5-day period (Figure 4). The growth rates of parental tumors (B16-F10) during this period were unaffected by any CD8+ T-cell treatment (data not shown). In contrast, growth of B16-SIY was completely arrested when treated with 2C-transduced CD8+ T cells (Figure 4b). As expected from historical analysis, m33-transduced CD8+ T cells were unable to slow the growth of B16-SIY tumors. CD8+ T cells transduced with either 2C or m33 libraries were also able to control of B16-SIY tumor growth. CD4+ T cells expressing the higher-affinity m33 and m33 library were both capable of slowing early growth of B16-SIY (Figure 4b), whereas CD4+ T cells transduced with 2C or the 2C library did not show significant effects on tumor growth.

Figure 4. Antigen-specific delay in tumor growth from T cells transduced with 2C or m33 TCR libraries. (a) Tumor growth rate for mice implanted with B16-F10 parental tumors, measured at day 5 post T-cell transfer shows no effective response across all transduced T-cell types. (b) Tumor growth rate for mice implanted with B16-SIY tumors, measured at day 5 post T-cell transfer. For CD8+ T cells, 2C, 2C-library and m33-library T cells were able to significantly reduce growth of the antigen-bearing SIY tumor compared with mock CD8+ cells (2C CD8+ P = 0.01, 2C-library CD8+ P = 0.02 and m33-library CD8+ P = 0.02) or m33 T cells, which had very little effect. In CD4+ T cells, m33 and m33 library significantly reduced tumor growth compared with mock-transduced CD4+ T cells (m33 CD4+ P = 0.01 and m33-library CD4+ P = 0.02), whereas wt 2C CD4+ and 2C library CD4+ did not differ from mock.

Figure 5. Frequencies of TCR 46β residues in TCR library-transduced T cells isolated from tumor and other tissues (in vivo selection). (a) Comparison of relative frequencies of TCR 46β residues selected for binding SIY/Kb in vitro (left panels) with TCR 46β residues isolated from B16-SIY tumors in mice treated with CD8+ (middle panels) or CD4+ (right panels) T cells transduced with the 2C (top row) or m33 (bottom row) libraries. The averages of two independent mice are shown, and data for individual mice can be seen in Supplementary Figure 3. For the 2C library in CD8+ T cells, analysis of signature residues (Met, Phe, Trp, Tyr and His) appearing in the tumor showed that Tyr, Phe, Met and His were significantly enhanced (P < 0.001). Signature residues located in the tumor from the m33 library in CD8+ T cells that are significantly enhanced are Phe, Met and His (P < 0.001) compared with a significant reduction of residues Tyr, Phe, Met, His and Trp in the tumor of m33 library CD8+ T cells (P < 0.001). (b) Relative frequencies of TCR 46β residues for T cells isolated from the draining LN (first and third columns) or spleen (second and fourth columns) of B16-SIY tumor-bearing mice treated with CD8+ (left two columns) or CD4+ (right two columns) T cells transduced with the 2C TCR library (top row) or the m33 TCR library (bottom row). The averages of two independent mice are shown for all samples, except for the 2C CD8+ library in the draining LN (6629 sequences; one mouse) and spleen (8809 sequences; one mouse), and for the m33 CD8+ library in the spleen (15400; one mouse). There is a significant enhancement of Phe, Trp (P < 0.001) and Tyr, His (P < 0.01) found in the tumor-draining LN from the 2C library in CD8+ T cells while Tyr, Phe and His from the same library were significantly enhanced in the spleen (P < 0.001). For the 2C library in CD4+ T cells, a significant reduction of residues Tyr, Phe, Met and His was found in the tumor-draining LN (P < 0.001). For the m33 library in CD8+ T cells, there is a significant reduction of residues Tyr, Phe, Met, His and Trp found in the tumor-draining LN (P < 0.001). Graphs for individual mice averaged in (a) and (b), along with the number of sequences analyzed per sample are shown in Supplementary Figure 3. A statistical approach to determine significance of amino-acid selections in vivo is described in Supplementary Figures 4 and 5. The approach uses the frequencies of all 20 amino-acid frequencies and the single-stop codon for each in vivo sample, in comparison with the in vitro SIY/Kb-selected frequencies.
example, $K_D$ value of about 3 μM for the m33 Y46βA mutant. These affinities are still considerably higher than the wild-type 2C, and it is these TCRs that account for the control of B16-SIY tumor growth with the m33 library in CD8+ T cells (Figure 4b).

Tumor-infiltrating CD4+ T-cell libraries express residues with diverse TCR affinities including highest-affinity TCRs CD4+ T cells with both the 2C and m33 libraries also infiltrated the B16-SIY tumor. Thus, to examine the impact of TCR affinity on redirecting CD4+ T cells to a class I tumor antigen, transcripts
from TILs of 2C and m33 libraries were sequenced. In this case, CD4⁺ T cells with the 2C library showed no obvious skewing (Figure 5a, Supplementary Figure 3a), consistent with a weak response by this diverse collection of low-affinity TCRs (Figure 4b). Nevertheless, the selective infiltration of these cells compared with the mock-transduced CD4⁺ T cells suggests that some of the cells migrate and are retained in the tumor owing to antigen specificity. CD4⁺ T cells transduced with the m33 library showed a distinct emergence of TCRs with the SIY/Kβ 'binding signature' (Figure 5a, Supplementary Figures 3b and 5), similar to that observed with CD8⁺ T cells expressing the 2C library. Thus, control of tumor growth by the m33 CD4⁺ T cells (Figure 4b) was associated with skewing of TCR residues at position 46β toward higher-affinity SIY/Kβ binding, whereas CD8⁺ T cells expressing these same TCRs were absent in the tumors.

Peripheral T cells with transduced TCRs have affinity distributions similar to TILs

The TCR library approach provides the opportunity to examine the influence of exogenous TCRs on peripheral T-cell distribution and survival (for example, the impact of 2C or m33 binding to self-dEV8/Kb).41,42 To examine these issues, various lymphoid tissues from mice with adoptively transferred T cells were examined 5 days after transfer by 454 sequencing analysis: these included the draining lymph nodes (LN), non-draining LN and spleens from tumor-bearing mice (Figure 5b). For the 2C library, CD8⁺ T cells showed modest skewing toward 'signature binding' residues in draining LNs and spleen (Supplementary Figures 3c and 4). This suggests that the 2C TCRs, probably in synergy with CD8, influenced the distribution to these tissues or the overall survival of transferred T cells, perhaps through binding to self-dEV8/Kb or SIY/Kβ. However, CD4⁺ T cells showed a more random distribution of residues in the libraries with perhaps some skewing toward 'signature binding' residues in the draining LN (Figure 5b).

Results of m33 library sequencing showed clearly that exogenous TCRs could determine the fate of peripheral T cells. In CD8⁺ T cells, m33 TCRs with the SIY/Kβ 'binding signature' residues had disappeared from LNs and spleen, consistent with our recent finding that m33 transduced into CD8⁺ T cells also disappeared rapidly from the periphery (Figure 5b, Supplementary Figures 3d and 5).33 The magnitude of the disappearance (~1 on a log scale) was equivalent to a 10-fold reduction in the m33 CD8⁺ T cells for each of the 46β variants, Met, Phe, Trp, Tyr and His. Interestingly, m33 TCRs with the Met at 46β were among those that disappeared, yet this m33 variant was not identified in the in vitro affinity selection (Figure 5a). This result suggests that the in vitro selection condition (2 nM SIY/Kβ-Ig) differs from the threshold for TCR-mediated deletion in vivo. Presumably, the binding interactions that are associated with in vivo deletion involve self-peptide/Kβ complexes such as dEV8/Kb, which for m33 have an affinity several orders of magnitude below the interactions with SIY/Kβ.17 As the binding difference between 2C and m33 for dEV8 is only about twofold, it is clear that the threshold for in vivo deletion is quite narrow.

As with the 2C library in CD4⁺ T cells, there was no obvious skewing of the variants from the m33 library in CD4⁺ T cells within the draining LNs or spleen (Figure 5b). The data suggest that the Trp variant may have shown a two- to threefold increase in frequency in both draining LNs and spleen, but this observation remains to be tested further.

T cells with single amino-acid substitutions at position 46β reveal distinct activity profiles depending on the presence of CD8 or CD4 co-receptor

To provide a more thorough view of the SIY-specific in vitro activation profiles of individual TCR variants, we transduced CD4⁺ and CD8⁺ T cells with several TCR variants and assayed them for activity. In addition to the wild-type TCRs with a Tyr at 46β, we chose both 'binding signature' variants and variants that did not bind with higher affinity to SIY/Kβ. As described, the variants possess binding affinities that range from 30 nM (for example, m33 Y46βI) to lower than 300 μM (for example, Y46βA of 2C).

To examine the TCRs at the lower range of affinities, retrovirus vectors encoding tyrosine (wt), alanine, phenylalanine, methionine, glycine or leucine at 46β for the 2C TCR were transduced into CD8⁺ (Figure 6a) or CD4⁺ (Figure 6b) T cells from C57BL/6 mice. Transduced cells were stimulated with: (1) anti-CD3, (2) SIY-pulsed antigen-presenting cell line T2-Kb, (3) the null peptide OVA-pulsed antigen-presenting cell line T2-Kb or (4) transduced cells alone. We have shown previously that in the presence of CD8, TCRs can exhibit Kβ values as low as 300 μM yet still retain significant peptide-specific activity.43 In line with this phenomenal ability of CD8 to synergize with very low-affinity TCRs, all of the 2C variants, including the alanine variant, were stimulated by SIY/Kβ (Figure 6a). These results are consistent with the ability of the CD8⁺ T cells transduced with the 2C library to infiltrate the tumor (Figure 3a), and to exhibit significant SIY-specific antitumor effects (Figure 4b).

In contrast to CD8⁺ T cells, the CD4⁺ T cells transduced with the same 2C TCR variants showed very low activity (wild-type Tyr; Phe) or no activity (Ala, Met, Gly and Leu) (Figure 6b, note the different scale). Thus, the 2C TCR is near the threshold for CD4⁺ T-cell activity, and is suboptimal for induction of these cells. Interestingly, the minimal activity associated with a few of the variants in the library must be adequate to drive SIY-specific infiltration (Figure 3a); however, as described above, many of the inactive variants in the library may be recruited to the tumor in an antigen non-specific process.

To examine the TCRs at the higher range of affinities, retrovirus vectors encoding tyrosine, alanine, phenylalanine, valine, histidine or isoleucine at 46β for the m33 TCR were transduced into CD8⁺ (Figure 6c) or CD4⁺ (Figure 6d) T cells from C57BL/6 mice. These TCRs exhibit affinities in the range of 30 nM (Tyr) to at least 3 μM (Y46βI), which is still 10 times higher affinity than the 2C wild-type TCR. As we have shown previously with the m33 TCR in a CD8⁺ T-cell hybridoma,50 the m33 TCR in CD8⁺ T cells mediated activation by self-peptide/MHC, even in the absence of SIY or OVA, although the level of activation was increased in the presence of SIY peptide (Figure 6c). Similarly, CD8⁺ T cells expressing m33 TCRs with other 'binding signature' residues (Phe and His) also showed self-peptide/MHC reactivity. These results are consistent with the finding that CD8⁺ T cells with these TCRs (m33 Tyr, Phe and His at 46β) were deleted in vivo (Figure 5). The three variants, Ala, Val and Ile, did not show significant stimulation with self-peptide/MHC in vitro, although they were stimulated effectively by SIY. Furthermore, these variants were not deleted in vivo, suggesting that in vitro self-peptide MHC activation results provide a useful surrogate indicator of in vivo CD8⁺ T-cell deletion.

CD4⁺ T cells transduced with these same m33 TCR variants showed very strong, SIY-specific activity, although the Val variant was somewhat reduced compared with the others (Figure 6d). These results indicate that almost all of the m33 variants would be superior to the wild-type 2C TCR in mediating CD4⁺ T-cell activity, consistent with the prediction that they have higher affinities for SIY/Kβ than the 2C TCR. These results also explain why the m33 library was superior to either 2C wt or 2C library in the control of the B16-SIY tumor (Figure 4b).

DISCUSSION

The system described here, with TCR libraries exhibiting a wide range of affinities for a class I pepMHC ligand, provides an opportunity to rapidly assess the TCR-binding properties associated with various T-cell processes. TCR libraries transduced into
polyclonal CD8\(^+\) T cells revealed the importance of choosing TCRs with affinities below a critical deletion threshold; T cells with TCRs above this threshold were rapidly eliminated. This process occurred with wt m33 in the absence of SIY antigen.\(^{13}\) While CD8\(^+\) T cells with the m33 ‘binding signature’ residues were eliminated, those variants with reduced affinity (for example, alanine) remained. These ‘lower’ affinity m33 variants have still considerably higher affinity than the wt 2C TCR, and were fully capable of mediating antigen-specific CD8\(^+\) T-cell activity and tumor inhibition. Perhaps related to the effects with the m33 ‘binding signature’ residues were found in antigen-bearing tumors on day 5 (Figure 3) and there was no infiltration of T-cell populations in the parental tumor B16-F10, which lacks the SIY antigen, on day 5 (data not shown). This result contrasts with a previous finding that showed trafficking of gp100-specific CD8\(^+\) T cells to both a gp100-positive tumor and a gp100-negative tumor implanted contralaterally.\(^{46}\) Differences in T-cell activation status could possibly account for the differences between the two studies; T cells in the gp100 study were transgenic and activated in vivo with gp100 vaccine and repeated high doses of IL-2, whereas our study used polyclonal T cells, activated in vitro with anti-CD3/anti-CD28 beads and lower doses of IL-2 followed by transduction with antigen-specific TCRs before transfer; neither a vaccine or IL-2 were included in our study. It is also possible that the antigen specificity of T-cell infiltration varies among tumor models. For example, bioluminescence imaging studies using different tumor models showed only antigen-specific CD8\(^+\) T-cell infiltration into EL4 tumors, but non-specific infiltration into MCA-205 tumors.\(^{47}\) The non-specific infiltration into antigen-negative MCA-205 tumors showed delayed kinetics compared with the antigen-positive tumors. In our model, we examined tumors only at day 5, so it is possible some infiltration in the B16 parental tumor might have occurred later. As such, it will be important to examine if the extent or kinetics of T-cell infiltration differ depending on these conditions (that is, tumor model, transgenic vs transduced T cells and activating conditions) and perhaps even between CD4\(^+\) and CD8\(^+\) T cells.

For clinical application of this therapy, the ideal scenario would be to introduce a single TCR with an affinity that is optimal for activity in both CD4\(^+\) and CD8\(^+\) T cells. The TCR library approach described here suggests that the optimal affinities will differ for...
CD4⁺ and CD8⁺ T cells, but that if a single TCR is to be chosen, for use in both CD4⁺ and CD8⁺ T cells; it would be the one that exhibits an affinity above 2C but below m33. Such a candidate TCR would include the alanine variant of m33 that exhibited potent CD4⁺ and CD8⁺ T-cell activity in vitro, no detectable self-reactivity in CD8⁺ T cells, minimal in vivo deletion in CD8⁺ T cells and persistence in tumors and peripheral lymphoid organs at day 5. Efforts are in progress to examine the activity of this variant compared with the wild-type 2C and m33 TCRs.

The design principles of the TCR libraries described here can be extended to other TCRs, as it is now clear that one or a few specific CDR1 or CDR2 residues provide significant binding energies in the interactions with the MHC-restricting elements. Accordingly, these residues not only influence binding to the antigen-pMHC ligand, but they determine the in vitro properties of T cells that depend on interactions between the TCR and self-pepMHC (for example, in persistence and homeostatic proliferation).

This is indeed why the TCRs with ‘binding signature’ residues in the m33 library were deleted, presumably by binding to self-pepMHC, such as dEV8/K6. Consistent with the hypothesis that Tyr46 of the Vβ8 region evolved to bind MHC, there was clear selection for TCRs with this residue (that is, binding selection based on in vitro experiments, and in vivo CD8⁺ T-cell deletion in the periphery).

However, amino acids at this position with alternative, conserved side chains (for example, Phe) also mediated efficient binding indicating that there is not a strict evolutionary selection for only tyrosine at this position. In this regard, it is also relevant to point out that this approach (TCR libraries) will allow the analysis of in vivo MHC-based selections to be examined without having to examine single-site mutations one at a time, as has been done previously.58

The studies described here also raise the possibility that different T-cell subsets will be optimally recruited with TCRs of different affinities against the class I MHC antigen. Accordingly, TCRs with different affinities might: (1) mediate distinct effector functions, (2) direct T cells toward different lineages, or (3) control memory T-cell development. In this regard, recent studies have revealed that TCR/class II MHC affinity has a role in the induction of Foxp3,50 yet the relationships of TCR affinity with each of these properties (especially in a CD4⁺, class I-specific system) remain to be determined. The TCR library approach described here provides a system to dissect these issues. Finally, it should be possible to use higher-affinity human TCRs as templates for the approach,59 to point out that this approach (TCR libraries) will allow the analysis of in vivo MHC-based selections to be examined without having to examine single-site mutations one at a time, as has been done previously.58

In vitro sorting of highest-affinity TCRs

58⁻⁺⁻ T-cell hybridomas were transduced with m33 wild-type TCR or the m33 Vγ6Yδ6 library, and 58⁻ co-expressing CD3βζ were transduced with 2C wild-type TCR or the 2C Vδζ library. Sorted under sterile conditions on a BD FACSAria (BD, Franklin Lakes, NJ, USA) for transformation. Resulting transformants were plated for 4 weeks in 24-well plates in 1 ml of fresh T-cell media. Each plate was washed with 1 ml of anti-CD3/anti-CD28 mouse T-activator Dynabeads (Invitrogen) in addition to 30 μl of recombinant mouse IL-2 (Roche, Basel, Switzerland) for 24 h. After Dynabead removal, activated T cells (in 1 ml) were added to a 24-well plate precoated with 1 μg/ml of Retronectin (Takara, Otsu, Shiga, Japan) followed by 1 ml of retroviral supernatant with an additional 20 ml of recombinant mouse IL-2. The plate was then centrifuged at 2000 g for 1 h at 30 °C. After centrifugation, the plate was incubated at 37 °C, 5% CO₂ for 48 h before analysis of TCR transgene expression by fluorescence-activated cell sorting via Vβ8.1.2 (BD Pharmingen, San Jose, CA, USA). CD4 or CD8 expression was confirmed using anti-CD4-APC-conjugated antibody clone RM4-5 (BD Pharmingen) or anti-CD8-APC-conjugated antibody clone 53-6.7 (eBioscience, San Diego, CA, USA).

Sequences from C57BL/6 mice harvested and passed through a 100-μm filter to obtain a single-cell suspension. Following red blood cell lysis, either CD8⁺ or CD4⁺ T cells were isolated from the splenocyte population by negative selection using the mouse CD8b⁻ or CD4 T-cell Isolation Kit II (Miltenyi, Bergisch Gladbach, Germany). A total of 10⁻⁶ isolated cells were plated per well in a 24-well dish in 1 ml of fresh T-cell media. Each well was washed with 1 ml of anti-CD3/anti-CD28 mouse T-activator Dynabeads (Invitrogen) in addition to 30 μl of recombinant mouse IL-2 (Roche, Basel, Switzerland) for 24 h. After Dynabead removal, activated T cells (in 1 ml) were added to a 24-well plate precoated with 1 μg/ml of Retronectin (Takara, Otsu, Shiga, Japan) followed by 1 ml of retroviral supernatant with an additional 20 ml of recombinant mouse IL-2. The plate was then centrifuged at 2000 g for 1 h at 30 °C. After centrifugation, the plate was incubated at 37 °C, 5% CO₂ for 48 h before analysis of TCR transgene expression by fluorescence-activated cell sorting via Vβ8.1.2 (BD Pharmingen, San Jose, CA, USA). CD4 or CD8 expression was confirmed using anti-CD4-APC-conjugated antibody clone RM4-5 (BD Pharmingen) or anti-CD8-APC-conjugated antibody clone 53-6.7 (eBioscience, San Diego, CA, USA).

Sequencing of TCR transcripts

To isolate total RNA from a T-cell suspension or tumor tissue, up to 10⁸ cells were pelleted (for T-cell suspension of 58⁻⁻⁻⁻ hybridomas) or up to 10⁹ mg of tissue were dissociated (for tissue). TRIzol RNA extraction was carried out on the pelleted cells or dissociated tissue using the PureLink RNA Mini Kit (Invitrogen). From isolated RNA, total complementary DNA (cDNA) was prepared using the Quantitect Reverse Transcription Kit (Qiagen). TCR Vb8 genes were amplified from the total cDNA using sequence-specific primers that harbored adapters and sequencing primers and done under sterile conditions on a BD FACSAria (BD, Franklin Lakes, NJ, USA), collecting the 1.4% top binding cells for both the 2C and the m33 library. The cells were expanded briefly in culture after sorting, followed by RNA isolation and sequence analysis (see below).

Selection of CD4⁺ TCRs

Selection from T-cell receptor libraries by the host

AS Chervin

Materials and methods

TCR retrovirus constructs and libraries

The TCR gene, codon optimized for expression in murine T cells (GeneArt, Regensburg, Germany), was cloned in as 2C—Ig—2C or m33x using the Not I restriction site at the 5’-end and EcoRI restriction site at the 3’-end into the MP71 retroviral vector. MP71 contains the LTR (long terminal repeat) from the myeloproliferative sarcoma virus and an improved 5’-untranslated sequence from the murine embryonic stem cell virus.56 For construction of the libraries, all oligonucleotide primers were ordered from Integrated DNA Technologies (IDT, Coralville, IA, USA). Two sets of primers were used in the construction of the library at position 46 of the CDR2ζ. Primer set 1 Forward 5’-CAAGCTCTACCAAGGGCGGCACTAGTACCAACACC-3’; Primer set 1 Reverse 5’-GTTGACATCGTCATGCTGGCGGCTGGTGCCTG-3’. Primer set 1 contains the oligonucleotide primer with the degeneracy (underlined) at position 46; Primer set 2 Forward 5’-ACCGGCAGGCTGAGTATGCTACCCNASGATCGGGCTCGAGACCCAGGAAGGG-3’; Primer set 2 Reverse 5’-GACCACGACCGACCAATCGACCG-3’. The two products from these reactions are then used to create the library product using the forward primer from set 1 and the reverse primer from set 2 by splice-overlap extension. The resulting PCR product from the second PCR reaction can be sequenced to ascertain if diversity exists at codon 46.I6

The PCR product containing the library was cloned into either 2C or m33-MP71 using the In-Fusion reaction kit (Clontech, Mountain View, CA, USA) via the Not I and BspE I restriction sites. Product from the In-fusion reaction was added to Turbo Competent—High Efficiency E. coli (New England Biolabs, Ipswich, MA, USA) for transformation. Resultants transformants were plated on LB Amnacillin and grown overnight at 37 °C at which time DNA was isolated from individual colonies (Qiagen, Valencia, CA, USA) to confirm library diversity at position 46 in the CDR2ζ.

Retrovirus production and transduction of T cells

The retroviral packaging line, Plat E 57 was plated at 4 x 10⁶ cells per 10-cm dish in Dulbecco’s modified Eagle medium (supplemented with 10% FCS, 2 mM l-glutamine and pen/strep). Twenty-four hours after plating, cells were transfected with 40 μg of retroviral DNA (either wild-type or library) via Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Four hours post transfection, the transfection mixture was aspirated and Plat E washed once with T-cell media (IMDM—10% FCS, 2 mM l-glutamine, 50 μM 2-mercaptoethanol, pen/strep), aspirated and replenished with 6 ml of fresh T-cell media. Forty-eight hours post transfection, the supernatant containing the packaged virus was harvested and passed through a 0.45-μm syringe filter to remove any cellular debris/particulates.

Spleens from C57BL/6 mice were pelleted (for T-cell suspension of 58⁻⁻⁻⁻ hybridomas) or up to 10⁹ mg of tissue were dissociated (for tissue). TRIzol RNA extraction was carried out on the pelleted cells or dissociated tissue using the PureLink RNA Mini Kit (Invitrogen). From isolated RNA, total complementary DNA (cDNA) was prepared using the Quantitect Reverse Transcription Kit (Qiagen). TCR Vβ8 genes were amplified from the total cDNA using sequence-specific primers that harbored adapters and sequencing primers and done under sterile conditions on a BD FACSAria (BD, Franklin Lakes, NJ, USA), collecting the 1.4% top binding cells for both the 2C and the m33 library. The cells were expanded briefly in culture after sorting, followed by RNA isolation and sequence analysis (see below).
were incubated with: (1) 5 μg/C20 donkey anti-rabbit secondary Ab at a concentration of 7.5
phosphate-buffered saline/glycerol. Tissue sections were incubated with
expressed as (length × width2)/2. Mice euthanized 5 days after transfer of
mock transfectants. Individual P-values are given in figure captions. Selection of the highest binding TCR variants in vitro (Figures 2b and c) shows representative data from four independent experiments, while in vivo selections of TILs or T cells in various lymphoid tissues (Figure 5) show the average values of two mice, where TCR variant selection in individual mice is shown in Supplementary Figure 3, including the number of sequences analyzed from each tissue. Statistical significance of differences in residue frequencies after in vitro or in vivo selection (Figures 2 and 5) compared with preselection library frequencies from E. coli were calculated using a standard χ2 test of significance. Values for significance of individual TCR variant selection in tumors and tissues (P-values) are given in the figure legends for ‘binding signature’ residues in those tumors or tissues where the signature residues were significantly enhanced or reduced (see Supplementary Figures 4 and 5).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
We would like to thank Janie Frye for her assistance with RNA isolation, Tom Gajewski for his gift of B16-SIY, Barbara Pilas and Ben Montez in the University of Illinois Flow Cytometry Facility for support with in vitro cell sorting, and Alvaro Hernandez and Chris Wright at the University of Illinois High-Throughput Sequencing and Genotyping Unit for assistance with 454 sequence analysis. This work was supported by NIH grant CA097296 (to DMK and HS) and a grant from the Melanoma Research Alliance (to DMK). BE was supported by a Research Fellowship of the DFG. JOS was supported by the Samuel and Ruth Engelberg/Irvinton Institute Fellowship of the Cancer Research Institute.

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