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The Impact of TCR-Binding Properties and Antigen Presentation Format on T Cell Responsiveness

Adam S. Chervin, Jennifer D. Stone, Phillip D. Holler, Ailin Bai, Jianzhu Chen, and David M. Kranz

TCR interactions with cognate peptide-MHC (pepMHC) ligands are generally low affinity. This feature, together with the requirement for CD8/CD4 participation, has made it difficult to dissect relationships between TCR-binding parameters and T cell activation. Interpretations are further complicated when comparing different pepMHC, because these can vary greatly in stability. To examine the relationships between TCR-binding properties and T cell responses, in this study we characterized the interactions and activities mediated by a panel of TCRs that differed widely in their binding to the same pepMHC. Monovalent binding of soluble TCR was characterized by surface plasmon resonance, and T cell hybridomas that expressed these TCR, with or without CD8 coexpression, were tested for their binding of monomeric and oligomeric forms of the pepMHC and for subsequent responses (IL-2 release). The binding threshold for eliciting this response in the absence of CD8 (K_D = 600 nM) exhibited a relatively sharp cutoff between full activity and no activity, consistent with a switchlike response to pepMHC on APCs. However, when the pepMHC was immobilized (plate bound), T cells with the lowest affinity TCRs (e.g., K_D = 30 μM) responded, even in the absence of CD8, indicating that these TCR are signaling competent. Surprisingly, even cells that expressed high-affinity (K_D = 16 nM) TCRs along with CD8 were unresponsive to oligomers in solution. The findings suggest that to drive downstream T cell responses, pepMHC must be presented in a form that supports formation of appropriate supramolecular clusters. The Journal of Immunology, 2009, 183: 1166–1178.

Interactions between Ag-specific receptors (TCRs) on CTL and peptide-MHC (pepMHC)

plasmon resonance (SPR) (1–3). Perhaps as a result of the varying conditions, values reported for binding parameters of a given TCR: pepMHC interaction have differed, sometimes considerably (1). Nevertheless, there is general agreement that TCR:pepMHC affinity constants are relatively low, falling, for example, orders of magnitude below what is commonly found for reactions of Ags with affinity-matured IgG Abs, but within the range seen for Ag reactions with primary response Abs, especially IgM (4), whose encoding gene segments, like those for TCRs, are not subject to somatic cell mutation.

The presence of the CD8 coreceptor on most CTL enhances the apparent sensitivity of TCR:pepMHC reactions (5–7), in part at least because CD8 binds, albeit with low affinity and rapid kinetics (8), to an invariant region on MHC-I molecules (9, 10). Besides helping to stabilize TCR:pepMHC complexes (11), CD8 helps recruit the kinase Lck to TCR complexes that are bound to pepMHC-I and thereby enhances signal transduction from the liganded TCR and consequent T cell responsiveness (11–13). Some evidence suggests that CD8 participation may vary depending on the nature of the TCR:CD8 or TCR:pepMHC interaction (14–16).

Given all of these complexities, it is perhaps not surprising that there has been considerable debate over which parameter(s) of the TCR:pepMHC interaction is most important for initiating a T cell’s response to pepMHC. Some evidence indicates that affinity (equilibrium-binding constant, K_D) is critical (17–19), but other studies suggest that it is not affinity, but dissociation rate (1/τ_{off} or lifetime) of the TCR:pepMHC complex that plays the pivotal role (20–24). Other data have implicated the importance of molecular flexibility of the interaction (25, 26). Another unresolved parameter involves the form of the cell surface TCR complex that is necessary for triggering the T cell (e.g., monomeric TCR/CD8, oligomeric TCR/CD8, or supramolecular TCR/CD8 and adhesion molecules). Although early studies suggested that binding of

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4 Abbreviations used in this paper: pepMHC, peptide-MHC; PBST, PBS containing 0.05% Tween 20; pERK, phosphorylated ERK; scTCR, single-chain TCR; SPR, surface plasmon resonance; Ig, transgenic; WT, wild type; MSCV, murine stem cell virus.

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soluble pepMHC monomers could stimulate T cell activity with the participation of CD8 (27), subsequent evidence has revealed that multivalent binding is essential (28–31), and that observations of triggering by pepMHC monomers may be an artifact due to transfer of stimulatory peptides from the monomers to MHC on the surface of cells for re-presentation (32, 33).

The typical strategy followed to study the relevant binding parameters in TCR-pepMHC interactions involves testing T cells that express a particular TCR against panels of APC or target cells that display diverse pepMHC, whose binding parameters to that TCR differ. Such studies can be difficult to interpret because pepMHC complexes formed from different peptides may differ considerably in their stability. In addition, the binding affinities of TCR-pepMHC interactions are typically quite low, making precise binding measurements with altered peptide ligands more difficult (3). Furthermore, cell surface coreceptor CD8, with its rapid kinetics (8), may influence cellular interactions between TCR and pepMHC to varying extents (6, 16), complicating attempts to correlate TCR:pepMHC-binding parameters that are measured in vitro with CD8+ T cell responses.

In this study, we have approached these problems with a different strategy: using yeast display (34) and genetic engineering, we created a panel of TCRs that bind the same pepMHC complex (SIYRYYGL-Kb, called SIYR/Kb) with affinities that vary ~1000-fold and on- and off-rate constants that vary ~60 and 90-fold, respectively. We measured the IL-2 release by transduced CD8 coreceptor-negative T cell hybridomas, each expressing a different member of the TCR panel, in response to the same pepMHC (SIYR/Kb) on the same APCs (T2-Kb). Although these hybridomas lacked CD8, they could be made to express this coreceptor by transfection. Thus, we have been able to evaluate the TCR-mediated responses of these T cells in the absence and presence of CD8. In parallel with the cellular responses, the binding parameters of the various TCR:pepMHC interactions were measured by SPR, using recombinant TCR and SIYR/Kb. Furthermore, to more closely approach the multivalent TCR:pepMHC interactions at the T cell-APC interface, we examined the binding of SIYR/Kb tetramers to the T cell hybridomas. We thereby established a ruler for tetramer-binding titrations, in the absence of complexities associated with CD8 binding, that can be used to compare results of the same approach used widely in the analysis of CD8-positive cells.

Our findings show that the responses of the CD8-negative T cells to the pepMHC complex on APC were strongly and equally correlated with affinity (Kd) and off-rate (k2); and a sharp threshold in these parameters separated responsive from nonresponsive cells. Surprisingly, cells that expressed the highest affinity TCR (Kd, 16 nM) and that bound the pepMHC tetramers very strongly (off-rate with t1/2, 10 min) were virtually unresponsive to either pepMHC tetramers or pepMHC-Ig dimers in solution, but responded strongly to the pepMHC immobilized on plastic as monomers or tetramers. These and other differences between soluble, APC-bound, and plate-bound forms of the pepMHC suggest that to drive downstream T cell responses (IL-2 production), the pepMHC has to be presented in a form that allows the appropriate supramolecular clustering on the T cell.

Materials and Methods

Peptides and cell lines

SIYR (SIYRYYGL), OVA (SIINFEKL), and QL9 (QLSPFPFDL) peptides were synthesized by the Macromolecular Core Facility of the Section of Research Sources, Penn State College of Medicine. Peptides were purified by reverse-phase chromatography using a C-18 column; mass was confirmed by MALDI. Peptide quantification by amino acid analysis was performed at the Molecular Structure Facility, University of California. Ecpak 2-293 (BD Clontech) retroviral packaging cell line maintained in DMEM supplemented with 10% FCS, t-glutamine, penicillin, and streptomycin. T2-Kb, a TAP-deficient lymphoblastoid cell line transfected with mouse Kb, and the αβ-negative 587+ T cell hybridoma were maintained in RPMI 1640 complete medium (supplemented with 10% FCS, t-glutamine, penicillin, and streptomycin). The 587+ T cell hybridomas transfected with a plasmid to express the 2C TCR or the high-affinity m8 TCR that bound 2C-2 with QL9-L2 (35), with or without cotransfection of plasmids to express CD8α and CD8β (17), were maintained in RPMI 1640 complete medium plus additional resistance marker antibiotics (G418 to select for TCRαβ, hygromycin to select for CD8α, and puromycin to select for CD8β). The 2C T cells were obtained by isolation of splenocytes from 2C TCR transgenic (tg) mice and stimulated in vitro at 4 × 106 cells/ml in RPMI 1640 complete medium containing 1 μM SIYR peptide and 5% rat Con A supernatant. Cells were used in various pepMHC activation assays on day 4 after stimulation.

Protein expression and purification

Single-chain Vb-linker-Va TCR constructs were cloned into the pET28a expression plasmid and transformed into BL21(DE3) (Stratagene) (36). Proteins were purified from inclusion bodies, which were added to 400 ml of denaturing solution (3 M urea, 2 mM reduced glutathione, 0.2 mM oxidized glutathione (pH 8)) at 4°C. Denaturation buffer (200 mM NaCl, 50 mM Tris (pH 8)) was added dropwise to the stirring mixture over a 36-h period. Ni-NTA agarose beads (Qiagen) were added to the refolding mixture for 24 h and collected using a scinttered glass funnel. Elution buffer (500 mM imidazole, 10 mM HEPES, 150 mM NaCl, 2.5 mM EDTA (pH 7.4)) was added to beads, and eluted TCR was purified over a Superdex 200 gel filtration column (GE Healthcare).

H2-Kb H chain containing a C-terminal biotinylination signal peptide and mouse or human β2-microglobulin light chains were expressed in E. coli. H2-Kb H chain was biotinylated in vivo by coinduction of biotin ligase, so that the H chain carried a biotin tag (37). Both chains were expressed as inclusion bodies, solubilized in urea, and refolded in vitro in the presence of excess peptide. Folded complexes were purified by anion exchange chromatography using HiTrap Q columns (GE Healthcare) and size exclusion chromatography. MHC complexes were incorporated into fluorescent tetramers for staining and dissociation experiments by adding streptavidin-PE (BD Pharmingen) stepwise to the purified, biotinylated MHCs to a final 1:4 molar ratio. BD dimers H-2Kb and H-2Lb IgG1 dimers (BD Pharmingen) were loaded with 40-fold molar excess peptide (SIYR and QL9, respectively) at 37°C overnight. T cell activation assays using these proteins, either immobilized on plastic or in solution, were conducted in the presence of 10 μM excess peptide.

Binding measurements of soluble receptors

Kinetic and equilibrium-binding data were obtained by SPR using a BIAcore 3000. Biotinylated SIYR/Kb and OVA/Kb monomers were immobilized on a neutravidin-coated CMS sensor chip on different flow cells to 400 response units. Soluble single-chain TCRs (scTCRs), purified by size exclusion chromatography no more than 24 h before measurements to avoid aggregates, at various concentrations in Biacore buffer (20 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20 (pH 7.4)), were flowed over the SIYR/Kb and OVA/Kb flow cells at 30 μl/min. Binding of scTCRs to the null complex OVA/Kb was subtracted from TCR binding to SIYR/Kb to correct for bulk shift and any nonspecific binding. All measurements were made at 25°C. On-rate, off-rate, and kinetic-based Kd analysis were performed using BIAevaluation 3.0 software. Equilibrium Kd values were calculated by Scatchard analysis. All measurements were performed two to five times.

Retroviral transductions

Restriction sites were added to the ends of 2Cα, m33α, and 2Cβ cDNAs by PCR, and the products were cloned into the retroviral vector murine stem cell virus (MSCV) at BgII/BstXI or BstXI/XmaI sites in a bicstronic configuration of 2Cα-IRES-2Cβ or m33α-IRES-2Cβ. Single-site alanine mutations were cloned into m33 MSCV using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). Transfection of the retroviral packaging cell line was conducted using the CalPhos Mammalian Transfection Kit (BD Clontech). Ecpak 2-293 (BD Clontech) packaging cells were transfected with 4 μg of MSCV DNA added to the CalPhos Mammalian Transfection Kit. Forty-eight hours posttransfection, retroviral-containing supernatants were collected, filtered, and added to 587+ T cell hybridoma cells with 8 μg/ml...
polybrene (Sigma-Aldrich). Cells were centrifuged at 1200 × g for 45 min, placed in a 37°C 5% CO2 incubator for 3 days, and assayed for TCR Cβ expression using biotinylated H57-597 (BD Pharmingen) and streptavidin-PE (BD Pharmingen). Positive TCR Cβ populations were sorted using a Cytomation MoFlo (DakoCytomation).

**T cell activation assays**

TCR-transduced 58־/־ cells (10^5) were incubated with one of various stimuli: immobilized SIYR/Kβ or QL9/Lα protein complexes, either as monomers, BD DimerX IgG1 dimeric fusions, or streptavidin-linked tetramers, immobilized anti-CD3 Ab (BD Pharmingen) at 5 μg/ml, or 10 T2-Kβ cells with various concentrations of SIYR peptide. Transduced cells were incubated with the various stimuli for 24 h at 37°C 5% CO2, and then supernatants were collected. For IL-2 detection, 96-well plates (Immunol 2HB) were coated with 2.5 μg/ml anti-murine IL-2 (BD Pharmingen) in 0.1 M Na2HPO4 (pH 9) for 2 h at room temperature, then blocked with 1% BSA in PBS for 12 h at 4°C. Wells were washed once with PBS containing 0.05% Tween 20 (PBST) before addition of 50 μl of PBST, and then 6.7 μg/ml biotinylated anti-murine IL-2 (BD Pharmingen) in PBS was added for 1 h at room temperature. Wells were washed three times with PBST, and then incubated with a 1/10,000 dilution of streptavidin-HRP substrate (Kirkegaard & Perry Laboratories) until a color change was seen in the control wells. The reaction was stopped with 50 μl of 1 N H2SO4, and absorbance at 450 nm was measured in each well using an EL_800 universal microplate reader (Bio-Tek Instruments). Activation experiments detecting IFN-γ were performed similarly to IL-2 detection, but supernatants were analyzed by IFN-γ ELISA (E Bioscience).

**Tetramer-binding and dissociation experiments**

For tetramer staining, 58־/־ cells transduced with 2C TCR mutants were incubated with various concentrations of streptavidin-PE SIYR/Kβ tetramer on ice for at least 2 h in the dark. After washing, cells were resuspended in ice-cold PBS containing 1% BSA and 0.02% azide, and analyzed for bound fluorescent tetramers by flow cytometry. The parental 58־/־ cell line was used as a control, and background fluorescence levels were subtracted from the TCR transfectant values at the same staining condition.

Tetramer dissociation experiments were performed, as described previously (38-40). Briefly, 58־/־ cells transfected with mutant TCR chains were stained with 293 nM (for m33, N27/Kβ, Y48/A, S51/A, and Y26/A) or 5.85 μM (for Y48/A) streptavidin-linked SIYR/Kβ tetramer, respectively, on ice for 2 h. Cells were washed and suspended in dissociation buffer containing 2% FCS, 0.1% azide, 100 μM cytosine arabinoside, and either with or without 20 μM Kβ blocking Ab (B.A.K-243) in RPMI 1640. At various times, cells were diluted in ice-cold PBS containing 1% BSA and 0.02% azide and analyzed by flow cytometry. Complete dissociation was determined to be the level of staining observed for the parental 58־/־ cell line.

**Measurement of intracellular phosphorylation of ERK(1/2)**

Cells were incubated with various stimuli at 37°C for 30 min. Cells were then fixed via addition of paraformaldehyde to a final 2% concentration, and then stained with anti-phospho-p44/42 Ab on ice (ERK(1/2), Thr202/Tyr204; Cell Signaling Technology). The cells were incubated with a 1/10,000 dilution of streptavidin-PE SIYR/Kβ tetramer (BD Pharmingen). Positive TCR Cβ populations were sorted using a Cytomation MoFlo (DakoCytomation). For tetramer staining, 58־/־ cells were transduced with 2C TCR mutants were incubated with various concentrations of streptavidin-PE SIYR/Kβ tetramer on ice for 0.5 h at room temperature. Plates were washed three times with PBST, and then incubated with a 1/10,000 dilution of streptavidin-PE SIYR/Kβ tetramer in PBS for 12 h at 4°C. Wells were washed once with PBS containing 0.05% Tween 20 (PBST) before addition of 50 μl of PBST, and then 6.7 μg/ml biotinylated anti-murine IL-2 (BD Pharmingen) in PBS was added for 1 h at room temperature. Wells were washed three times with PBST, and then incubated with a 1/10,000 dilution of streptavidin-PE SIYR/Kβ tetramer on ice for at least 2 h in the dark. After washing, cells were resuspended in ice-cold PBS containing 1% BSA and 0.02% azide, and analyzed for bound fluorescent tetramers by flow cytometry. The parental 58־/־ cell line was used as a control, and background fluorescence levels were subtracted from the TCR transfectant values at the same staining condition.

**Results**

**Generation of a panel of 2C TCR mutants**

The high-affinity 2C TCR mutant m33 was generated through directed evolution of the CDR3α, using a single-chain (Vβ-linker-Vα) form of the wild-type (WT) receptor in a yeast display system (34). To examine the affinity threshold for CD8 independence more precisely and to explore the role of binding kinetics and multimerization in T cell activation, we generated a panel of receptors with affinities for SIYR/Kβ (measured by SPR) that varied between 2C WT (K_D = 30 μM) and m33 (K_D = 16 nM). To accomplish this, single-site alanine mutations were introduced into the high-affinity TCR m33. These mutations were guided by two previous papers in which we performed alanine scans of CDR residues in the WT 2C TCR, examining their binding to SIYR/Kβ (41) and QL9/Lα (42). In the 2C WT TCR, the chosen mutations yielded a range of effects on pMHC binding, and we anticipated the same might be the case if these mutations were cloned into the higher affinity m33 mutant. Seven mutant TCRs carrying single alanine mutations in either the Vα or Vβ were chosen as follows: N27/Kβ, N30/A, Y48/A, Y26/A, Y49/A, Y50/A, and S51/A (supplemental Fig. 1). The single-site alanine mutant receptors were each introduced into the m33 yeast surface display vector, and the expression of properly folded scTCR on the yeast was confirmed in each case using a Vβ-specific Ab (anti-Vβ8.2 mAb F23.2; data not shown). The mutants were also assayed for ability of the yeast cells displaying them to be stained with SIYR/Kβ tetramers (supplemental Fig. 2a). Under the conditions used, the tetramers stained yeast expressing m33 and four of the mutants (Y26/A, S51/A, Y49/A, and N27/Kβ), but not WT 2C and three other mutants (Y50/A, N30/A, and Y48/A). The levels of tetramer staining correlated with the magnitude of the binding effects observed in the original alanine scan of the 2C TCR (supplemental Fig. 2b). Accordingly, we examined in more detail the binding properties of all nine TCRs and their ability to mediate T cell activity.

**Binding properties of 2C TCR mutants**

To measure the equilibrium-binding and kinetic (on-off-rate) constants for their reaction with SIYR/Kβ, each TCR was cloned into E. coli and expressed as a soluble single-chain protein (36) for use in SPR (Fig. 1a). The affinities of scTCR forms of 2C WT and m33 (K_D values of 30 μM and 16 nM, respectively) were found to be similar to the affinities previously measured by this method for full-length 2C WT (43) and full-length m33 (34). The m33 mutants displayed affinities ranging from 2C WT at the low end, and extending to near m33 at the high end (Table I). S51/A and Y26/A displayed high affinities for SIYR/Kβ similar to m33 (K_D values of 15 and 17 nM, respectively); m33 bound with the fastest on-rate (1.37 × 10^6 M^-1 s^-1), and S51/A with the slowest off-rate of the panel (τ_1/2 almost 90 s). For the weakest binding TCRs (2C and Y50/A), dissociation was extremely rapid (τ_1/2 ~ 1 s). The single-site mutations yielded differences in binding affinity among the panel, but there was not a significant difference in the intermediate affinity range (K_D ~ 1 μM). In hopes of generating such a mutant, we cloned the double mutation S51/A/Y48/A, with the idea that the slight increase in affinity of the S51/A mutation (compared with m33) would be additive with the Y48/A mutation. This was indeed the case because the S51/A/Y48/A mutant exhibited a K_D value of 540 nM.

It has been proposed that, at least for some TCRs, interactions with the helices of the MHC influence predominantly the binding on-rate, whereas TCR interactions with the peptide influence the stability (off-rate constant) of the complex (44). In this context, one might have expected CDR1 and CDR2 mutations in residues that contact the Kβ helices to affect predominantly the on-rate. However, these data do not support this mechanism because there was no apparent correlation between CDR mutant location and their effects on the on-rate or off-rate constants. Collectively, the TCR mutants showed changes in on-rate, off-rate, or both (Table I).

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* The online version of this article contains supplemental material.
FIGURE 1. Analysis of TCR mutants by SPR. a, Measurements were performed on a Biacore 3000. Biotinylated SIYR/Kb and OVA/Kb monomers were immobilized to a neutravidin-coated CM5 sensor chip on separate flow cells at equal response units. Soluble TCRs were flowed over sensor chip at various concentrations, and binding was measured by change in response units. Nonspecific binding to the null ligand OVA/Kb was subtracted from final measurements. Kinetic analysis was measured using BIAEvaluation 3.0 software. All measurements were done at 25°C. Two of the mutant TCRs (S51/αA/Y48/βA and N30/βA) exhibited residual binding at the end of the dissociation curves; however, equilibrium-binding constants calculated from kinetic measurements were consistent with those determined under equilibrium conditions using Scatchard plots (Table I and b). b, Scatchard analysis was performed on the SPR data to give an equilibrium $K_D$ value for each receptor. Bound TCR on the x-axis was plotted against bound TCR divided by concentration of TCR on the y-axis and fitted by linear regression. Equilibrium affinity was calculated as the negative reciprocal slope of this line. $K_D$ values derived from kinetic parameters and equilibrium analyses were in good agreement (Table I).
### Table I. Binding properties of soluble scTCR

<table>
<thead>
<tr>
<th>TCR</th>
<th>$k_i$ (M$^{-1}$s$^{-1} \times 10^{-3}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
<th>$t_{1/2}$ (s)</th>
<th>$K_d/K_s$ (nM)</th>
<th>Equilibrium $K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S51</td>
<td>515 ± 2</td>
<td>0.008 ± 0.001</td>
<td>86 ± 12</td>
<td>15 ± 2</td>
<td>30 ± 13</td>
</tr>
<tr>
<td>m33</td>
<td>1,370 ± 670</td>
<td>0.015 ± 0.003</td>
<td>46 ± 10</td>
<td>16 ± 12</td>
<td>22 ± 20</td>
</tr>
<tr>
<td>Y26</td>
<td>850 ± 420</td>
<td>0.013 ± 0.001</td>
<td>50 ± 3</td>
<td>17 ± 8</td>
<td>24 ± 12</td>
</tr>
<tr>
<td>N27</td>
<td>520 ± 30</td>
<td>0.021 ± 0.002</td>
<td>33 ± 4</td>
<td>40 ± 7</td>
<td>42 ± 8</td>
</tr>
<tr>
<td>Y49</td>
<td>280 ± 140</td>
<td>0.012 ± 0.001</td>
<td>58 ± 2</td>
<td>47 ± 23</td>
<td>74 ± 57</td>
</tr>
<tr>
<td>S51/A/Y48pA</td>
<td>136 ± 74</td>
<td>0.076 ± 0.048</td>
<td>11 ± 7</td>
<td>543 ± 56</td>
<td>545 ± 70</td>
</tr>
<tr>
<td>Y49pA</td>
<td>136 ± 35</td>
<td>0.35 ± 0.18</td>
<td>2 ± 1</td>
<td>2,900 ± 2,100</td>
<td>1,900 ± 1,100</td>
</tr>
<tr>
<td>N30</td>
<td>34 ± 11</td>
<td>0.27 ± 0.06</td>
<td>3 ± 1</td>
<td>8,200 ± 900</td>
<td>6,600 ± 1,500</td>
</tr>
<tr>
<td>Y50</td>
<td>241 ± 175</td>
<td>1.3 ± 0.3</td>
<td>0.5 ± 0.1</td>
<td>6,950 ± 3,750</td>
<td>5,900 ± 1,800</td>
</tr>
<tr>
<td>2C(T)</td>
<td>20.4 ± 3.4</td>
<td>0.72 ± 0.06</td>
<td>0.93 ± 0.14</td>
<td>35,600 ± 710</td>
<td>23,800 ± 6,000</td>
</tr>
</tbody>
</table>

All experiments were carried out by SPR at 25°C. SIYR/K$^b$ monomer was immobilized to sensor chip, and soluble scTCR was flowed in solution. Equilibrium affinity calculated from the negative inverse slope of Scatchard analysis.

**SIYR/K$^b$ tetramer binding to CD8-negative T cell hybridomas**

Given our unique panel of TCRs with diverse monovalent (SPR-based) binding affinities for pepMHC, it was of interest to characterize the multivalent binding of pepMHC tetrators to these TCRs on T cells. The binding of pepMHC to T cells is, however, inherently complicated by the participation of CD8 in the binding reactions (6, 7). Hence, each of the mutant TCR subunits was cloned into full-length m33-based binding affinities for pepMHC, it was of interest to characterize TCR:pepMHC binding is to measure pepMHC streptavidin

tetramer off-rate from the surface of T cells (38, 39). Again, this approach is complicated by the participation of CD8 in the dissociation reaction. To directly correlate with the monovalent TCR-binding affinities and kinetics, SIYR/K$^b$ tetramer dissociation rates were measured for various T cell lines in the absence of CD8 (Fig. 2c). TCR-transduced cells were incubated on ice for 2 h with tetramer concentrations that yielded approximately equal levels of bound tetramers (290 nM for higher binding TCRs, including m33, S51,A, Y26,A, N27pA, and Y49pA). After washing the cells (at 4°C) to remove unbound tetramers, cells were suspended at 24°C in dissociation buffer, which contained anti-K$^b$ Ab to block rebinding of dissociated tetrators and cytochalasin D and sodium azide to minimize tetramer internalization. At various times, the dissociation was stopped by dilution with ice-cold buffer, and the samples were analyzed by flow cytometry. For each receptor tested, a t$_{1/2}$,tet was calculated by fitting a single exponential curve to the dissociation data (Fig. 2c and Table II). This value correlated strongly with the monovalent t$_{1/2}$, measured by SPR (Fig. 2d). A previous study of the OT-1 TCR (monovalent K$_d$ = 6 µM, t$_{1/2}$ = 30 s (50)) attempted to minimize the effects of CD8 by using a K$^b$ mutant with reduced CD8 binding; it showed a dissociation rate (t$_{1/2}$) for the tetramer of OVA/K$^b$, which is a strong agonist for the OT-1 TCR, of 80 s at 37°C (38). By comparison, the SIYR/K$^b$ tetramer dissociated relatively rapidly from the Y48pA TCR (t$_{1/2}$ = 13 s at 24°C; Table II), which binds the SIYR/K$^b$ monomer relatively weakly (by SPR monovalent K$_d$ = 2.9 µM, t$_{1/2}$ = 2 s; Table I).

**T cell activity of 2C TCR mutants with APCs**

To assess the functional capabilities of the transduced CD8-negative T cell lines, we measured their production of IL-2 in response to APCs (T2-K$^b$) that were incubated with various concentrations of SIYR peptide. Five of the cell lines (expressing the TCRs N27pA, Y49pA, S51,A, Y26,A, and m33) responded well, whereas cell lines that expressed four of the receptors (Y50,A, N30pA, Y48pA, and 2C) were not stimulated at any concentration of SIYR tested (Fig. 3a). T cells expressing TCR S51/A/Y48pA showed an intermediate level of activation (Fig. 3a). The concentrations of SIYR peptide that yielded half-maximal activity (SD$_{50}$ values) were remarkably similar for the five responding cell lines, but there was roughly a two order of magnitude reduction in potency for T cells expressing the S51/A/Y48pA receptor (Fig. 3b).

Importantly, all of the cell lines displayed a similar ability to respond by IL-2 production to an immobilized (plate-bound) anti-CD3 Ab (supplemental Fig. 4a).
When the same 10 TCRs were transduced into hybridomas that also expressed CD8, every cell line was stimulated to produce IL-2 in response to both anti-CD3 Abs and SIYR/Kb (Fig. 3, c and d, and supplemental Fig. 4b). Accordingly, as expected, T cells expressing the four receptors (Y48/H9252A, N30/H9252A, Y50/H9251A, and 2C) that were not stimulated in the absence of CD8 were active when transduced into CD8-positive cells (Fig. 3d). The SDso values of these lines were slightly more varied than the responsive CD8-negative lines, and some of the variability could have been due to modest differences in CD8 expression levels (data not shown) rather than differences in TCR-binding properties. The effect of the expression levels of coreceptor, which are known to vary on the surface of T cells (reviewed in Ref. 51), on the apparent sensitivity of a CD8-dependent cell line is well known (52) and emphasizes the importance of considering CD8 levels in such systems. CD8 expression in the high-affinity, CD8-independent T cells resulted in only a modest increase in sensitivity (i.e., lower SDso values) because of a threshold determined by the minimal number of SIYR/Kb complexes presented on the surface of APCs. As to be expected from the correlations between equilibrium affinity and kinetic constants, no distinction could be made with this panel of receptors as to the more relevant parameter for TCR triggering. Notably, however, the results further support the view that even TCRs with slow dissociation rates (e.g., S51/H9251A) can stimulate robust T cell activity. Conversely, in the presence of coreceptor, even TCR:pepMHC interactions with fast dissociation rates can trigger potent responses.

T cell activity of 2C TCR mutants with various forms of purified, recombinant pepMHC

To further explore the signaling competency of TCRs on the CD8-negative cells, we stimulated them with either soluble or immobilized (plate-bound) recombinant SIYR/Kb MHC monomers and tetramers. In the initial experiments, SIYR/Kb streptavidin-linked tetramers were adsorbed on the surface of plastic tissue culture...
wells before the addition of T cells, and the subsequent IL-2 response was measured. Unexpectedly, this stimulus was able to specifically trigger all of the TCR transductants in the panel, including those for which no stimulation was seen with peptide-pulsed APCs (Fig. 4, a and b). Although the CD8-negative T cell responses to peptide-pulsed APCs largely showed SD50 values that amounted simply to positive or negative results without much differentiation between them (Fig. 3 a), the SD50 values for the cells’ responses to immobilized SIYR/Kb were more widely dispersed (Fig. 4, a and b); nevertheless, they again correlated strongly with both \( K_D \) and off-rate (\( t_{1/2} \)) values measured for the receptors by SPR (Fig. 4, c and d, compared with Fig. 3, e and f). IL-2 release was not observed when the cells were stimulated with the null pepMHC complex OVA/Kb immobilized on tissue culture plates (Fig. 4 a), or when 58/H11002/H11002 cells expressing a TCR with a different specificity (m6 35)) were stimulated with immobilized SIYR/Kb tetramer (Fig. 4 a).

When equivalent mass concentrations of SIYR/Kb monomers (1 \( \mu \)M) and tetramers (0.25 \( \mu \)M) were used to coat plastic wells, the plates were equally stimulatory for hybridomas that expressed high-affinity TCR m33, whether or not the CD8 coreceptor was also expressed (Fig. 5, a and b). Although there was some decrease

### Table II. Multivalent binding properties of cell surface-expressed TCR

<table>
<thead>
<tr>
<th>TCR</th>
<th>( K_{D,SPR} ) (nM)</th>
<th>( K_{D,tet} ) (nM)</th>
<th>Enhancement Factor</th>
<th>( t_{1/2} ) (s)</th>
<th>( t_{1/2,tet} ) (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S51/A</td>
<td>15 ± 2</td>
<td>0.96</td>
<td>15.6</td>
<td>86 ± 12</td>
<td>906 ± 198</td>
</tr>
<tr>
<td>Y26/A</td>
<td>17 ± 8</td>
<td>1.4</td>
<td>12.1</td>
<td>50 ± 3</td>
<td>354 ± 72</td>
</tr>
<tr>
<td>m33</td>
<td>16 ± 12</td>
<td>1.56 ± 0.43</td>
<td>10.2</td>
<td>46 ± 10</td>
<td>510 ± 42</td>
</tr>
<tr>
<td>N27/A</td>
<td>40 ± 7</td>
<td></td>
<td>33 ± 4</td>
<td>222 ± 12</td>
<td></td>
</tr>
<tr>
<td>Y49/A</td>
<td>47 ± 23</td>
<td></td>
<td>58 ± 2</td>
<td>816 ± 42</td>
<td></td>
</tr>
<tr>
<td>S51/A/Y48/A</td>
<td>540 ± 56</td>
<td>14</td>
<td>38 ± 11</td>
<td>27.6 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>Y48/A</td>
<td>2.900 ± 2,100</td>
<td>26</td>
<td>111</td>
<td>2 ± 1</td>
<td>13.2 ± 4.2</td>
</tr>
<tr>
<td>2C WT</td>
<td>23,800 ± 6,000</td>
<td>11 ± 1</td>
<td>2,160</td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) All measurements correspond to TCR interactions with SIYR/Kb. \( K_D \) values were measured by SPR using scTCRs (Table I). Multimeric \( K_D \) measured from titration of SIYR/Kb-PE tetramers to TCR-transduced 58/H11002/H11002 T cell hybridomas (\( K_{D,tet} \)).

Enhancement factor \( = \frac{K_{D,SPR}}{K_{D,tet}} \), SIYR/Kb tetramer off-rate (\( t_{1/2,tet} \)) values measured in presence of anti-Kb Ab B.8.24.3.
in stimulation when SIYR/Kb was immobilized as a monomer rather than a tetramer for the 2C WT TCR (Fig. 5, c and d), both formats were able to stimulate the cells. These results suggest that it is the multivalent arraying of immobilized, closely packed ligands, rather than their special close proximity in tetramers, that was responsible for the cell response.

There has been some controversy in the literature about the ability of soluble pepMHC monomers, or even tetramers, to stimulate T cell activity. Having a collection of high-affinity TCRs, we were in a position to test the ability of these reagents to stimulate a late, downstream response (IL-2 secretion) under conditions in which a high proportion of the surface TCRs bound the pepMHC ligand. This system is also advantageous in that the transduced T cell lines do not express the cognate MHC (Kb) that would enable re-presentation of dissociated peptide (SIYRYYGL) from the soluble pepMHC (32, 33). Soluble MHC monomers at the same concentration or higher than was used to prepare the plate-bound (immobilized) monomers provided no stimulation, even for
cells with the high-affinity TCR m33, in which SIYR/Kb monomer would be at saturating levels (Fig. 5, a and b). Perhaps more surprisingly, little or no IL-2 release was observed in response to soluble SIYR/Kb tetramers even for cells that expressed CD8 co-receptor (Fig. 5, a and b). The same lack of activity was observed at various concentrations of the soluble tetramer (data not shown).

We further explored this phenomenon with soluble IgG1 fusion MHC dimers to ensure that the lack of stimulation with the streptavidin reagent was not merely a limitation of the tetramer geometry. In this system, we used both SIYR/Kb and the well-known alloantigen QL9/Ld that stimulates both the WT 2C TCR (K<sub>D</sub> = 1.5 μM) (53, 54) and the high-affinity m6 TCR engineered against this complex (K<sub>D</sub> = 14 nM) (35, 54). The 2C and m33 T cell hybridomas that did or did not coexpress CD8αβ were incubated with 10 nM (1.25 μg/ml) SIYR/Kb IgG1 fusion dimer either pre- or immobilized on the surface of plastic tissue culture wells, or maintained as a soluble stimulus for 24 h at 37°C. Supernatants were analyzed for IL-2 secretion. As was seen for the peptide-MHC tetramer, immobilized dimeric ligand was quite potent at stimulating a cytokine response, whereas soluble dimeric ligand was unable to stimulate IL-2 release (Fig. 6a). This behavior was also observed when QL9/Ld-Ig fusion dimer was used to stimulate T cell hybridomas carrying either the WT 2C TCR or the high-affinity m6 TCR, with or without CD8αβ (Fig. 6b). As with soluble pepMHC tetramer, a titration of either soluble SIYR/Kb-Ig fusion or soluble QL9/Ld-Ig fusion did not stimulate T cells to release IL-2 (data not shown). Studies to address the minimum size of an oligomeric complex that would stimulate these T cells are currently ongoing.

Finally, to assess whether normal effector T cells from a 2C tg mouse responded similarly, the QL9/Ld-IgG1 dimers were used. T cells from the 2C tg mice are H2-K<sup>b</sup> positive, but negative for H-2L<sup>d</sup>, so there is no possibility of ambiguous results based on re-presentation of the peptide (32, 33). The 2C tg T cells were stimulated with the alloantigen QL9/Ld (K<sub>D</sub> = 1.5 μM) by immobilized or soluble QL9/Ld<sup>d</sup> IgG1 dimers. Consistent with the results from the transduced T cell hybridomas, immobilized QL9/Ld<sup>d</sup> IgG1 dimer was able to stimulate IFN-γ release from the 2C tg T cells, whereas soluble dimer was ineffective (Fig. 6c). Results were identical if a 10-fold higher concentration of QL9/Ld<sup>d</sup> IgG1 dimer was used in the assay (data not shown).

Activation efficiency differences associated with TCR affinity

It was of some interest to note that the magnitude of the maximum tetramer staining (Fig. 2a) varied with the TCR-binding affinity in the same way that the efficiency of IL-2 release varied upon stimulation with immobilized SIYR/Kb tetramers (Fig. 4a). In particular, the maximal tetramer-staining levels and the levels of IL-2 release were consistently lower for the CD8-dependent receptors than for the CD8-independent receptors.

In the tetramer titration experiments, the histograms of the fluorescently labeled cells followed a normal (Gaussian) distribution at each ligand concentration (data not shown). This homogeneity contrasts with activation of intracellular phosphorylation cascades that seem to follow a more bimodal on or off distribution when measured on CD8<sup>+</sup> T cells (55). We asked therefore whether the reduced responsiveness of our CD8-negative hybridomas that expressed the lower affinity TCRs, which required high levels of plate-bound SIYR/K<sup>b</sup>, resulted from the response of only a small fraction of the cells or from a weaker response by all cells in the entire population. To investigate this issue, we monitored phosphorylation of ERK in response to immobilized stimuli by flow cytometry, allowing us to look at the responses of individual cells. We incubated T cells with either plate-bound anti-CD3 or SIYR/K<sup>b</sup> for 30 min and then measured the level of phosphorylated ERK (pERK) (56). The pERK response under these conditions clearly showed the response was indeed bimodal; only a relatively small fraction of cells responded in cases where IL-2 secretion was low (Fig. 7). The fraction of pERK-positive 2C T cells
cells stimulated by immobilized SIYR/K<sup>B</sup> was ~40% of the fraction responding to anti-CD3, consistent with the relative levels of total IL-2 detected in supernatants of the same cells (Fig. 4A). In general, the percentage of pERK-positive cells corresponded to previous IL-2 secretion experiments: responses to SIYR/K<sup>B</sup> were improved by addition of CD8 coreceptor, expression of a higher affinity TCR (m33), or stimulation with immobilized anti-CD3 Ab.

**Discussion**

By creating a panel of TCR molecules that bind the same pepMHC ligand with a broad range of affinities and off-rates (t<sub>1/2</sub>), we have been able to examine in unprecedented detail the influence of t<sub>1/2</sub> and affinity of the TCR:pepMHC interaction on a T cell activation response (IL-2 production). For each member of the TCR panel, we characterized its binding to the pepMHC ligand as the following: 1) a monovalent reaction, using a single-chain form of the TCR and SPR; and 2) a multivalent reaction, by analyzing the binding of pepMHC tetramers to the TCRs expressed on T cell hybridomas. The binding parameters of these interactions were then correlated with the IL-2 secretion response of these hybridomas, as either CD8<sup>−</sup> or CD8<sup>+</sup> cells, to the pepMHC on APCs (T2-K<sup>B</sup>). The results of the monovalent (SPR) reactions are summarized in Table I, and the multivalent (tetramer) reactions in Table II.

The most illuminating results were obtained with the T cell hybridomas that lack CD8. Those cells whose TCRs had an affinity for the pepMHC above a threshold value (delineated by the affinity of the double-mutant TCR SS1<sub>A</sub>/AY48<sub>B</sub>/A, K<sub>D</sub> < 550 nM (Figs. 1–3)) made robust responses to the pepMHC on the APCs, whereas those whose TCR affinity was below this threshold did not respond. This abrupt affinity threshold of T cell activity resembles the sharp TCR:pepMHC affinity threshold recently described for thymic negative selection by Daniels et al. (38). Although the reasons differ, these all-or-none (digital) responses represent a marked departure from the graded (analog) responses that generally characterize T cell responses as well as Ab-Ag reactions. It should be noted that the threshold characterized in this paper is for the secretion of IL-2, a relatively late event in the effector T cell response (57). Other activities, such as increased intracellular calcium ion concentration, cell surface CD69 up-regulation, cytotoxicity, or T cell proliferation, could be governed by different affinity thresholds (58). Ultimately, in vivo T cell expansion and effector responses will also presumably be determined by some optimal affinity of the TCR:pepMHC interaction (59), perhaps influenced by additional innate factors (60).

The monovalent and multivalent binding of SIYR/K<sup>B</sup> by the double-mutant S51<sub>A</sub>/AY48<sub>B</sub>/A were only slightly better than by the single-site mutant Y48<sub>B</sub>/A (Figs. 1 and 2; Table I), but strikingly, responsiveness to pepMHC on APCs was observed for the double mutant but not for Y48<sub>B</sub>/A, even when the peptide concentrations were orders of magnitude higher (Fig. 3A). Hence, we conclude that the binding properties for S51<sub>A</sub>/AY48<sub>B</sub>/A lie very close to the threshold for coreceptor-independent stimulation by APCs, and may be used to define a tipping point between activating and non-activating TCRs in the absence of CD8.

There has been much debate as to which parameter of the TCR:pepMHC-binding reaction (K<sub>D</sub> vs k<sub>d</sub> or t<sub>1/2</sub>) is the most important determinant of T cell responses. In our system, because both K<sub>D</sub> and t<sub>1/2</sub> values were directly related to each other for every TCR, we were unable to decisively address the most relevant parameter. A less than consistent correlation between T cell responsiveness and TCR:pepMHC t<sub>1/2</sub> has often been noted before (reviewed in Ref. 61). In some cases, TCR-pepMHC interactions can elicit T cell responses despite their having short t<sub>1/2</sub> values, and these cases were associated with relatively large changes in heat capacity on forming TCR:pepMHC pairs (25). To account for the findings, it has been proposed (26) that constraints on the molecular flexibility of TCR and pepMHC when anchored in their respective cell membranes could result in interactions having significantly longer t<sub>1/2</sub> than when the same TCR and pepMHC interact in SPR, where they behave essentially as though in solution, under conditions in which most TCR-pepMHC k<sub>d</sub> values are measured. Along with increased valency effects, these flexibility-related constraints could contribute to the slow dissociation of MHC tetramers from the surface of CD8-negative T cells as opposed to the rapid dissociation measured by SPR (Fig. 2, c and d, and Table II).

For the T cells that expressed the same TCR, but also CD8, the sharp difference between the high-affinity responders and the low-affinity nonresponders was obscured because in the presence of CD8 the latter also responded (Fig. 2, a and c). This effect of CD8 is in accordance with the general rule that for TCR-mediated T cell responses, the enhancing effect of the coreceptor is much more apparent for weak than strong TCR:pepMHC reactions (6, 17). Although the affinity threshold for activation of CD8<sup>−</sup> cells was not addressed in this study, CD8 could decrease the threshold considerably, because CD8<sup>+</sup> T cells have been shown to specifically lyse target cells that present p2Ca/K<sup>B</sup>, whose interaction with the 2C TCR has a K<sub>D</sub> = 0.3–1 mM (i.e., 10- to 30-fold lower than the 2C TCR affinity for SIY/K<sup>B</sup>) (53, 62).

FIGURE 7. pERK(1/2) responses to efficient and inefficient stimuli. Flow cytometry analysis of pERK levels in hybridomas that expressed the 2C TCR, or the 2C TCR + CD8αβ, or the m33 TCR each in response (30 min) to no stimulus or immobilized SIYR/K<sup>B</sup> monomer or immobilized α-CD3.
By eliminating the contribution of CD8 to pepMHC binding, we are able to show explicitly that the enhancement achieved by multivalency varies depending on the monovalent affinity. The enhancement is greatest at lower affinities, with 2C exhibiting a binding enhancement over 2000-fold with tetramer (Table II). This relationship of monovalent affinity to multivalent binding enhancement is analogous to binding studies in which monovalent and bivalent Ab fragments bound to ligands that were plate bound at high or low densities: the lower affinity Abs exhibited greater binding enhancement with the increase in ligand density compared with the higher affinity Abs (46, 48, 49). We think that a likely explanation involves the approximate 5-min washing step, wherein tetramers bound univalently will tend to be lost in proportion to their dissociation rate \( t_{1/2} \): for weak binders \( t_{1/2} \approx 1 \) s the only tetramers that survive the washing step are bound trivalently or possibly bivalently, whereas for strong binders \( t_{1/2} > 50 \) s more univalent and bivalently bound tetramers remain bound. Hence, tetramer binding and SPR binding are less divergent for the strong binders than the weak binders. This finding of varying multivalent enhancement factors points to the usefulness of multivalent binding assays to compare different TCR:pepMHC interactions, but it also suggests that quite small changes in intrinsic binding affinities could yield larger changes in multivalent binding.

We were surprised by the finding that CD8-negative T cells that expressed the very low-affinity TCRs were able to respond to the pepMHC complex immobilized on the surface of a plastic well, although they were completely unresponsive to SIYR/K\(^b\) on the surface of APCs. Preliminary efforts to quantitate the total densities of SIYR/K\(^b\) in each format suggested that they do not differ substantially (e.g., by orders of magnitude). Recent evidence indicates that class I pepMHC complexes on the surface of cells, although mobile, tend to linger in relatively immobile clusters around ICAM molecules, increasing the local concentration dramatically and improving the ability of T cells to scan those complexes (64). Thus, if anything, we might have expected our APCs to serve as a more efficient mechanism for the clustering of multiple pepMHC. It is possible that localized patches of more highly clustered pepMHC are achieved when immobilized on plastic surfaces than on the surface of an APC, but this remains to be seen. A similar observation about the potency of immobilized ligands, using CD8-positive T cells, led Ma et al. (65) to propose a deformation model of TCR triggering. Variability in immobilized surface density could, in part, explain the lower fraction of stimulated cells seen for lower affinity receptors (Fig. 7) (e.g., depending on where a cell settles, the local ligand density could either be above or below a critical threshold for stimulating the cell). Another difference between cell surface and plate-immobilized Ag presentation that may impact the cell response is the duration of the TCR’s presentation. The dynamics of pepMHC complex turnover expected on the surface of APC, which may result in changing Ag densities over the course of the 24-h assay (66), are absent when immobilized pepMHC are used as a stimulus.

It might reasonably have been predicted that T cells transduced with the highest affinity TCR m33, together with the coreceptor CD8αβ, would be efficiently stimulated with SIYR/K\(^b\) tetramers because this ligand should bring together multiple TCR and CD8 molecules into a cluster. Indeed, early studies showed that T cell hybridomas with lower affinity WT TCRs were stimulated by pepMHC multimerized on soluble dextran or agarose beads (67). Their study showed that multivalent interactions were important, but they were unable to determine whether this was because of the low affinity of normal TCR:pepMHC interactions, or a true requirement for extensive cross-linking. In the present study, despite highly efficient activation by immobilized SIYR/K\(^b\), there was little stimulation of IL-2 secretion induced by SIYR/K\(^b\) dimers or tetramers in solution (Figs. 5 and 6). This result was especially unexpected given that both the \( t_{1/2} \) and the equilibrium tetrameric binding enhancement factor \( \Gamma \) indicated that long-lived, multivalent binding takes place under these conditions, and it has been shown that soluble pepMHC oligomers with dimeric valency or higher can initiate early TCR recognition processes and early signaling events such as calcium influx even with WT relatively low-affinity receptors (28, 31, 68), in a mechanism that may involve the recognition of endogenous pepMHC complexes through CD8 engagement (5, 69). From our results, it seems likely that large order clustering of TCR complexes by agonist pepMHC, beyond that obtained with a streptavidin-linked tetramer, is needed to drive T cell signaling all the way to late effector functions, such as IL-2 secretion. The specific size and nature of a fully stimulatory pepMHC complex are unclear, but most likely lies between a tetramer and the larger order organization present when pepMHC complexes are immobilized at high density on a plate. These structures may need to be large enough to recapitulate some of the functions of the immunological synapse, such as providing a directionality for secretion (reviewed in Ref. 70).

Finally, the results presented in this study provide a guide toward the use of genetically engineered TCRs in adoptive T cell therapies (71–75), because one of the major goals of such studies is to engineer CD8-independent, class I MHC-restricted TCRs for introduction into CD4\(^+\) Th cells. Our previous studies with the m33 TCR have shown that by raising the affinity of the TCR for SIYR/K\(^b\) to 30 nM, transduced T cells showed self-reactivity (34). It is clear from the present study that efficient CD8-independent T cell targeting can be achieved with TCRs that have considerably lower affinity, possibly avoiding a degree of self-reactivity that is the result of cross-reactivity with structurally related self peptides (such as deEV8 in the 2C system).

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

References

Induced sensitization of tumor stroma leads to eradication of established cancer by T cells. J. Exp. Med. 204: 49–55.


Supplementary Figure Legends

Supplementary Figure 1. Residues mutated in the creation of the SIYR/K\textsuperscript{b} TCR panel.
The MHC-binding surface of the 2C TCR (1TCR in the PDB, Garcia, K.C. et. al. (1996). Science 274, 209-219.) is depicted with the main backbone shown as a cartoon (alpha in pink, and beta in blue), and mutated residues shown as space-fill models. Residues which differ between 2C WT and the high-affinity mutant m33 are shown in yellow. Residues which, when mutated singly to alanine from m33, result in CD8-independent activation by SIYR-pulsed antigen-presenting cells are shown in red. Residues which, when mutated singly to alanine from m33, result in receptors which cannot respond to SIYR-pulsed antigen-presenting cells in the absence of CD8 are depicted in green.

Supplementary Figure 2. Binding comparison between 2C and m33 alanine scans. (a) Yeast displaying panel of scTCR m33 mutants were stained with SIYR/K\textsuperscript{b} -PE tetramer and relative binding measured as mean fluorescent units (MFU). (b) Resulting MFUs were plotted against the log reactivity of the indicated alanine mutations in the wild-type 2C TCR binding to SIYR/K\textsuperscript{b} tetramers as determined by ELISA (41).

Supplementary Figure 3. TCR surface levels of and SIYR/K\textsuperscript{b} staining of 58\textsuperscript{-/-} TCR transduced cell lines.
58\textsuperscript{-/-} CD8-negative (a) and CD8-positive (b) hybridomas were retrovirally transduced with the panel of TCR mutants. Surface expression was checked with biotinylated H57-597 (anti-C\textbeta) and detected with streptavidin-phycoerythrin. The C\textbeta positive cells were enriched by fluorescence-
activated cell sorting (FACS). Values represent mean fluorescent intensities. (c) TCR-transduced T cell hybridomas without CD8 were stained with 200nM SIYR/K\textsuperscript{b} tetramer linked with streptavidin-phycoerythrin and analyzed by flow cytometry.

**Supplementary Figure 4. Biological activity of 58\textsuperscript{-/-} CD8-negative and CD8-positive transduced hybridomas.**

58\textsuperscript{-/-} CD8-negative (a) and CD8-positive (b) TCR transduced hybridomas were stimulated with 5 \textmu g/mL of plate bound 145-2C11 (anti-CD3) for 24 hours and assayed for IL-2 production.
Supplemental Figure 1

View toward TCR from perspective of MHC
Supplemental Figure 2

a

Yeast

Counts

10^3 10^4 10^5

Yeast

2.2
3.9
5.9
4.3
7.7
36.6
30.8
30.3
64.4
39.8

SIY/K^b-PE Tetramer

Control
2C
Y50\_A
N30\_A
Y48\_A
N27\_A
Y49\_A
Y26\_A
m33
m33
S51\_A

b

SIY/K^b Binding to m33 Mutants

SIY/K^b Binding to 2C Mutants

m33
S51\_A
Y49\_A
Y48\_A
N30\_A
N27\_A
Y50\_A
Y26\_A

Log SIY/K^b Binding to 2C Mutants
Supplemental Figure 3

a) CD8-Negative

- 58⁻⁻ : 4.7
- 2C : 208
- Y50αA : 187
- N30βA : 179
- Y48βA : 111
- N27βA : 177
- Y49αA : 234
- Y26αA : 112
- m33 : 215
- S51αA : 309

b) CD8-Positive

- 58⁻⁻ : 3.5
- 2C : 104
- Y50αA : 88
- N30βA : 66
- Y48βA : 24
- N27βA : 65
- Y49αA : 97
- Y26αA : 99
- m33 : 132
- S51αA : 112

c) CD8-negative

- 58⁻⁻ : 2.6
- 2C : 2.7
- Y50αA : 2.9
- N30βA : 2
- Y48βA : 3.3
- N27βA : 19
- Y49αA : 20
- Y26αA : 16
- m33 : 33
- S51αA : 23

TCRβ

SIY/Kb-PE Tetramer
Supplemental Figure 4

a  58^/- CD8-Negative

b  58^/- CD8-Positive

IL-2 Production (A_{450})