

CD8⁻ T Cell Transfectants that Express a High Affinity T Cell Receptor Exhibit Enhanced Peptide-dependent Activation

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Abstract

T cells are activated by binding of the T cell receptor (TCR) to a peptide-major histocompatibility complex (MHC) complex (pMHC) expressed on the surface of antigen presenting cells. Various models have predicted that activation is limited to a narrow window of affinities (or dissociation rates) for the TCR-pMHC interaction and that above or below this window, T cells will fail to undergo activation. However, to date there have not been TCRs with sufficiently high affinities in order to test this hypothesis. In this report we examined the activity of a CD8-negative T cell line transfected with a high affinity mutant TCR ($K_D = 10$ nM) derived from cytotoxic T lymphocyte clone 2C by in vitro engineering. The results show that despite a 300-fold higher affinity and a 45-fold longer off-rate compared with the wild-type TCR, T cells that expressed the mutant TCRs were activated by peptide. In fact, activation could be detected at significantly lower peptide concentrations than with T cells that expressed the wild-type TCR. Furthermore, binding and functional analyses of a panel of peptide variants suggested that pMHC stability could account for apparent discrepancies between TCR affinity and T cell activity observed in several prior studies.

Key words: T cell activity • dissociation rate • binding affinity • interleukin 2 • yeast surface display

Introduction

The α/β TCR recognizes a peptide bound to a product of the MHC. This recognition is programmed during thymic development, where a T cell will die if it does not bear a TCR that binds to a self peptide/MHC ligand or if it binds too tightly. These processes, known as positive and negative selection, yield a T cell repertoire that is MHC restricted but not self-reactive. That is, T cells must recognize only peptides that are bound to MHC molecules (rather than free peptides) and must have the potential to recognize only those peptides that appear “foreign”. “Foreignness” can be defined as either a difference in structure compared with the repertoire of normal self peptides or as a change in the level of the peptide that is present normally.

It has been shown that TCR-peptide-MHC (pMHC) binding affinities that distinguish the processes of positive selection and T cell activation exhibit an extremely narrow window (1). For example, affinities with K_D values of ~ 100 μM appear to be sufficient to deliver signals for positive selection, while affinities with K_D values of 1–10 μM elicit either negative selection or activation of peripheral T

cells (2). It has been suggested that it is the affinity of a TCR-pMHC interaction (3) or the dissociation rate of the TCR from the pMHC (4, 5) that determines the biological activity of a T cell. In the “kinetic proofreading model,” it has been proposed that TCR-pMHC lifetimes of dissociation ($t_{1/2}$) in the range of ~ 10 to 40 s yield full signaling and T cell activation, while shorter lifetimes yield partial signaling and antagonist effects (6–8). Lanzavecchia and colleagues have shown that a single pMHC complex appears to engage and downregulate up to 100 TCR molecules per T cell in a process they refer to as “serial triggering” (9). This serial triggering of TCRs has been proposed to be important in optimal signaling of the T cell. Based on this and the kinetic model it has been suggested that long lifetimes of dissociation might prevent serial triggering and thus signaling. It has also been proposed that a TCR-pMHC interaction might reach an “affinity ceiling,” above which there is no advantage for the T cell (10). This proposition was based largely on analysis of the 2C system, where the TCR has the highest measured affinity for a pMHC, the alloantigen QL9/L^d ($K_D \sim 0.1$ μM to 3 μM depending on the type of measurement; references 11 and 12). Only a few QL9/L^d complexes appeared to be necessary to trigger the 2C T cell and hence it is dif-

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difficult to reconcile how a higher affinity TCR could be even more effective (13).

The models described above predict that TCR affinities above a particular threshold will be detrimental to T cell activity or, at the least, will not yield an improvement in pMHC sensitivity. Potentially related to these possibilities, there have been a number of examples of T cells that have TCRs with higher affinity or slower off-rates for a pMHC, yet they exhibited reduced activity for the pMHC (1, 14–16). Of particular note is a recent study that examined two different class I-reactive T cell systems to support the notion that higher affinity (slower off-rates) for a pMHC yielded reduced activity (17). These studies were limited in that the affinities and off-rates reside within the narrow range that is typical of in vivo-selected T cell clones (i.e., K_D values that are in the micromolar range, with lifetimes of 10 to 40 s; reference 18).

To address this issue more conclusively, we have examined the activity of a T cell that expresses a TCR with 100-fold greater affinity than even the highest affinity interaction yet studied, the 2C TCR and QL9/L^d. The study was made possible by using the m6 mutant TCR from CTL 2C ($K_D = 10$ nM) that was engineered for higher affinity by a process of yeast surface display and directed evolution (19). The m6 mutant contains identical V α and V β regions as the 2C TCR except for the tip of the CDR3 α loop, which differs at five contiguous residues between m6 (HQGRY) and 2C TCR (GFASA). T cell hybridomas transfected with the m6 or wt TCR were compared for IL-2 release and TCR downregulation over a range of concentrations of QL9 and QL9 variants. The variants were selected based on their different binding affinities for the TCRs (as a peptide/L^d complex) and for their differences in binding to and stabilization of L^d. The findings indicate that: even with an affinity as high as 10 nM and a dissociation lifetime of 1,650 s, T cells exhibited enhanced, peptide-specific activity. This suggests that the kinetic or equilibrium “optimum” models need to be revised. We also observed that a peptide/L^d complex (p2Ca/L^d) that displayed high affinity for the m6 TCR yielded lower activity than might be predicted from the affinity. Evidence indicated that this reduced biological activity could be accounted for by the instability of the pMHC complex over the period of the assay. We propose that the analogous “aberrant” behavior (between TCR–pMHC affinity and activity) observed in various systems might be explained by pMHC stability differences, especially over the course of relatively long T cell activation assays.

Materials and Methods

Peptides. MCMV (YPHFMPTNL), p2Ca (LSPFPFDL), QL9 (QLSPFPFDL), and QL9 variant peptides were synthesized by standard F-moc chemistry at the Protein Sciences Facility at the University of Illinois, Urbana, IL, and were purified by C-18 reverse-phase HPLC using a linear acetonitrile gradient. Peptide purity and concentration were determined by quantitative amino acid analysis.

mAbs. KJ16 is a rat IgG mAb that is specific for the mouse V β 8.1 and V β 8.2 regions of the TCR. F23.1 is a mouse IgG2a mAb that binds to the mouse V β 8.1, 8.2, and 8.3 regions. F23.2 is a mouse IgG1 mAb that is specific for the mouse V β 8.2 region. 30–5–7 is a mouse IgG2a mAb that binds to the α 2 domain of the mouse MHC molecule, L^d. Abs were purified from ascites or culture supernatants.

Peptide–MHC Production. The J558L hybridoma that expresses the L^d/IgG1 fusion was provided by Dr. Sean O’Herrin, University of Chicago, Chicago, IL (20). Cells were grown in serum-free hybridoma media (GIBCO BRL) until they reached ~60–70% viability. Supernatants were then harvested, filtered (0.45 μ m), and concentrated in a stirred-cell concentrator (Amicon; 10,000 dalton molecular weight cut-off). Proteins were precipitated in 50% saturated ammonium sulfate, resuspended in 50 mM K₂HPO₄, pH 7.7, and purified by gel filtration HPLC (G-200; Amersham Pharmacia Biotech). Appropriate fractions were pooled, concentrated, and exchanged into peptide loading buffer (130 mM citrate, 150 mM NaCl, 124 mM Na₂HPO₄, pH 6.5). Specific peptides were loaded by incubating L^d/Ig at 37°C for 1.5 h in the presence of 100-fold molar excess of peptide. After incubation, the pH was adjusted to 7.4 with 1.0 M Tris, pH 9.0, and the complexes were allowed to refold at 4°C for 24 h. Purity was assessed by SDS-PAGE. Peptide-loaded complexes were purified a second time by G-200 HPLC immediately before use in BIAcore experiments.

Soluble TCR Production. The pRMHa-3 *Drosophila* expression plasmids containing the 2C α and β chains (with the transmembrane regions deleted) were provided by Dr. Luc Teyton, The Scripps Research Institute, La Jolla, CA (12). The 2C CDR3 α was mutated to the m6 CDR3 α by a two-step Quick-change method (Stratagene). The m6 α and 2C β pRMHa-3 plasmids were cotransfected together with a plasmid conferring G418 resistance (pUCHsNEO) into SC2 *Drosophila melanogaster* cells by Ca₃(PO₄)₂ precipitation (19:19:1 ratio α : β :NEO). Stable transfectants were selected and maintained at 27°C in Schneider’s complete media (GIBCO BRL) containing 1 mg/ml G418. TCR expression was induced by the addition of CuSO₄ to a final concentration of 500 μ M. Supernatants were harvested after 3 d, concentrated, and dialyzed against PBS, pH 8.0. His₆-tagged TCRs were isolated by native nickel affinity chromatography using a 5 ml Ni-NTA agarose (QIAGEN) column. Appropriate fractions were pooled, concentrated and purified further by gel filtration HPLC.

Peptide–MHC Binding Assays. T2-L^d is a human lymphoblastoid line transfected with the L^d heavy chain (21). To monitor TCR binding to cell-bound peptide/L^d, T2-L^d cells (~3 \times 10⁶/ml) were incubated with purified peptides (~1–10 μ M final concentration) at 37°C for 2 h. Cells were washed with PBS containing 1% BSA (PBS/BSA) and stained for 30 min on ice with 2C or m6 TCR *Drosophila* supernatants. Cells were washed with PBS/BSA and bound TCR was detected with biotinylated F23.1 IgG (F23.1:biotin) followed by streptavidin:phycoerythrin (SAV:PE; BD PharMingen). Cellular fluorescence was measured by flow cytometry on a Coulter Epics XL instrument. To measure TCR dissociation rates from cell-bound peptide/L^d, peptide-loaded T2-L^d were incubated with m6 TCR supernatants, washed, and resuspended at room temperature in PBS/BSA containing excess F23.2 IgG (~300 μ g/ml) as a competitor. At various times, cells were washed with ice cold PBS/BSA, centrifuged, and remaining cell-bound m6 TCR was detected with F23.1:biotin and SAV:PE as described above. Cellular fluorescence was measured by flow cytometry.

Surface plasmon resonance (SPR)* measurements were performed on a BIAcore 3000 instrument (BIAcore). Rabbit anti-mouse IgG was coupled to a CM5 surface through amine chemistry. Kinetic experiments were performed by immobilizing ~600 RU of QL9/L^d/Ig followed by injections of various concentrations of purified m6 TCR at a flow rate of 30 μ l/min in PBS at 25°C. Surfaces were regenerated with 10 mM glycine, pH 1.5. Responses were subtracted from a blank surface prepared by ethanolamine deactivation of the CM5 surface. Response curves were fitted to a 1:1 Langmuir binding model. TCR binding was not observed using the null peptide, MCMV/L^d/Ig, immobilized on the chip.

T Cell Transfections. Full-length 2C α and 2C β cDNAs were cloned into a modified form of the SFFV expression plasmid (22). The construct containing the α chain also contained a neomycin resistance gene. The 2C CDR3 α was mutated to the m6 CDR3 α by a two-step Quickchange procedure (Stratagene). NotI-linearized plasmids were electroporated at a 1:10 ratio (α : β) with 5×10^6 58^{-/-} cells. 58^{-/-} (CD4⁻, CD8⁻, K^{b-}, L^{d-}), a variant of the DO-11.10.7 mouse T cell hybridoma, does not express functional TCR α or β chains but does express the CD3 subunits (23). Approximately 24 h after transfection, cells were placed under antibiotic selection (1.5 mg/ml G418) and after 1–3 wk colonies were picked, expanded, and examined for TCR expression. Initial screening of colonies with F23.1 and F23.2 was performed in order to isolate transfectants that had approximately the same level of β -chain.

T Cell Activation Assays. TCR transfectants (7.5×10^4) were incubated with T2-L^d cells (7.5×10^4) and various concentrations of peptides in 96-well U-bottom plates at 37°C and 5% CO₂. After 30 h, supernatants were harvested and assayed for IL-2 by ELISA or with the IL-2-dependent cell line HT2 in a [³H]thymidine uptake assay. To monitor peptide/L^d stability and CD3/TCR downregulation, cells were stained with m6 TCR *Drosophila* supernatants followed by F23.1:biotin (or KJ16:biotin). The final incubation included SAV:PE (BD PharMingen) to detect F23.1 bound to the surface TCR on transfectants and the bound m6 TCR on the T2-L^d cells, anti-CD90.2 FITC (53–2.1, anti-Thy-1.2; BD PharMingen) to detect 58^{-/-} transfectants, and anti-CD3 ϵ -cychrome (2C11; BD PharMingen) as a second marker for TCR/CD3 levels. Cellular fluorescence was measured by flow cytometry, gating on CD90.2⁺ cells (TCR transfectants).

To assay IL-2 levels, ELISA plates (Immulon 2 HB; Dynex Technologies) were coated overnight at 4°C with 2.5 μ g/ml rat anti-mouse IL-2 IgG (JES6–1A12; BD PharMingen) in 0.1 M Na₂HPO₄, pH 9.0. Plates were washed with PBS–0.05% Tween 20 (PBS–T), blocked with PBS/1% BSA, and undiluted supernatants were added and incubated at room temperature for 1 h. After washing, plates were incubated with 7 μ g/ml biotinylated rat anti-mouse IL-2 IgG (JES6–5H4; BD PharMingen) for 1 h at room temperature. After washing, streptavidin-horseradish peroxidase (HRP; Kirkegaard and Perry Laboratories) was added at a dilution of 1:10,000 for 30 min. Plates were washed, TMB substrate (Kirkegaard and Perry Laboratories) was added, reactions were stopped with 1 N H₂SO₄, and absorbance (450 nm) was measured. Identical results were obtained when the IL-2-dependent cell line HT2 was used to monitor IL-2 levels in a [³H]thymidine uptake assay (data not shown).

*Abbreviations used in this paper: pMHC, peptide-MHC complex; SAV:PE, streptavidin-PE; scTCR, single-chain TCR; SPR, surface plasmon resonance; wt, wild-type.

Results and Discussion

Peptide/L^d Binding by the m6 Mutant TCR. To draw more conclusive correlates of affinity (or off-rate) and T cell activity, we have isolated higher affinity TCRs by a directed evolution strategy (19). In the present study, we elected to study the mutant m6 that showed the highest affinity for QL9/L^d. The m6 TCR was cloned into two different expression systems, yeast and insect, in order to produce soluble TCR. The yeast secreted m6 single-chain TCR (scTCR) (V β -linker-V α) has been described previously and exhibited a K_D of 9 nM as measured in a competition assay (19). In this assay, [¹²⁵I]-labeled Fab fragments of the anti-L^d antibody 30–5–7 were competed for binding by soluble wild-type (wt) 2C scTCR and m6 mutant scTCR. To further explore the affinity and kinetics of m6 TCR binding to various QL9 peptide variants (24), a soluble, full-length heterodimer of wt and m6 TCR were produced in *Drosophila* cells. The peptide variants included p2Ca (lacking the NH₂-terminal glutamine of QL9), or several QL9 variants containing position five (Phe) substitutions (QL9–Y5, QL9–M5, QL9–R5) or position eight (Asp) substitutions (QL9–E8 and QL9–Q8). Phe₅ of QL9 is predicted to be buried in the C pocket of L^d, (25) and position 5 variants are known to affect both TCR binding and L^d binding (24). Asp₈, on the other hand, is predicted to point directly toward the TCR, yielding a negatively charged surface at this position (25). The L^d-binding null peptide MCMV was used as a control. Soluble m6 and wt TCR binding was detected by loading the peptides under saturating conditions onto L^d expressed on the surface of T2-L^d cells and then incubating cells with the full length TCRs, followed by F23.1:biotin and SAV:PE. As shown in Fig. 1, m6 TCR exhibited a range of binding to these peptide-L^d

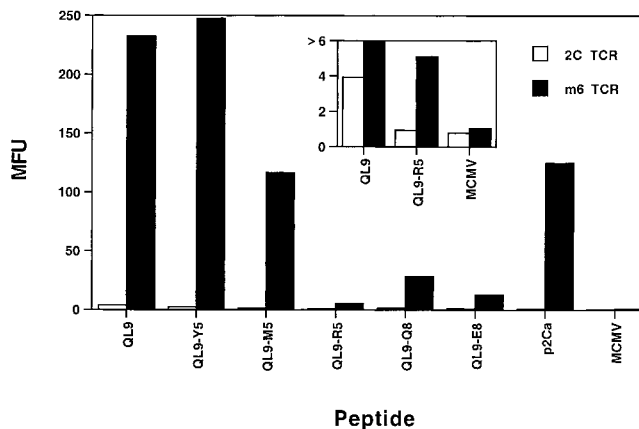


Figure 1. Soluble TCR binding to cell surface peptide/MHC. T2-L^d cells were loaded with saturating concentrations of peptides for 2 h at 37°C, washed, and then incubated at room temperature with *Drosophila*-expressed soluble 2C or m6 TCR followed by F23.1–SAV:PE. Cellular fluorescence was measured by flow cytometry. L^d-binding peptides included the null peptide MCMV (YPHFMPNTNL) and the 2C-reactive peptides p2Ca (LSPFPFDL), QL9 (QLSPFPFDL), and various QL9 variants that contained the indicated mutations (e.g., QL9–Y5 contains a Tyr substitution for the Phe at position 5).

complexes. The low end of the range (i.e., binding of m6 to QL9-R5/L^d; see inset, Fig. 1) approaches approximately that of the wt TCR–QL9/L^d interaction whereas the high end of the range (i.e., binding of m6 to QL9-Y5/L^d) is slightly improved compared with the m6 TCR–QL9/L^d interaction. The relative binding among these pMHC was identical at 4°C, 25°C, and 37°C (data not shown).

To quantitate the binding of m6 TCR to QL9/L^d and to examine binding kinetics, the m6 TCR–QL9/L^d interaction was measured by surface plasmon resonance using BIAcore instrumentation. QL9/L^d/Ig was immobilized on an anti-Ig sensor chip and m6 was injected at various concentrations (Fig. 2 A). The association constant (k_a) of m6 binding was 4.2×10^4 (M⁻¹s⁻¹) and the dissociation constant (k_d) was 4.2×10^{-4} (s⁻¹). The corresponding values for the wt 2C TCR are 0.6×10^4 (k_a in M⁻¹s⁻¹) and 200×10^{-4} (k_d in s⁻¹), as measured by BIAcore analyses (12). The affinity constant determined from these kinetic constants was 10 nM for m6, in agreement with the value of 9 nM determined for the m6 scTCR by a completely independent method. The affinity of the wt TCR for QL9/L^d was 3 μM based on BIAcore analyses (12) and 1.5 μM based on the competitive inhibition assay described above (19). Hence, the m6 TCR has approximately a sevenfold faster on-rate, a 45-fold slower off-rate, and a 300-fold higher affinity than the 2C wt TCR (Table I). If one compares the TCR–QL9/L^d lifetimes by calculating $t_{1/2}$ values ($t_{1/2} = \ln 2/k_d$), the m6 TCR exhibits a $t_{1/2}$ of 1,650 s, compared with that of wt 2C TCR which is 35 s. The lifetimes of TCR–pMHC interactions for strong agonists in several T helper cell systems typically exhibit $t_{1/2}$ values in the 10 to 15 s range (2, 5).

Because of the slow dissociation rate, we were also able to use a flow cytometry–based assay to measure dissociation of m6 from QL9/L^d and several QL9 variant/L^d complexes on T2-L^d cells (Fig. 2 B). In these assays, the average dissociation constant (k_d) for m6 from QL9/L^d was 8.5×10^{-4} (s⁻¹), in close agreement with BIAcore data. The average m6 dissociation constants for QL9-Y5/L^d (5.9×10^{-4} s⁻¹); QL9/L^d (8.5×10^{-4} s⁻¹), p2Ca/L^d (1.2×10^{-3} s⁻¹), and QL9-M5/L^d (5.0×10^{-3} s⁻¹) were completely consistent with the qualitative binding analysis shown in Fig. 1. The $t_{1/2}$ values calculated from these data (QL9/L^d, 830 s; QL9-Y5/L^d, 1,180 s; p2Ca/L^d, 580 s; QL9-M5/L^d, 140 s) were all at least 10 times longer than the lifetimes of normal TCR–pMHC interactions for strong agonists.

Role of Affinity in T Cell Activation. Genes encoding the 2C β-chain and either the wt α chain or the m6 α chain were cotransfected into the 58^{-/-} T cell hybridoma that lacks its own αβ TCR but contains the CD3 subunits and thus is fully capable of undergoing TCR–mediated activation (23). Clones of G418-resistant transfectants from the 2C wt TCR and m6 TCR were isolated and examined for TCR surface levels using the Vβ-specific Abs F23.1 and F23.2, which bind to nonoverlapping epitopes. The clonotypic antibody 1B2 could not be used because the CDR3α mutations in m6 eliminate binding of this antibody. Eight colonies each of wt and m6 TCR that were

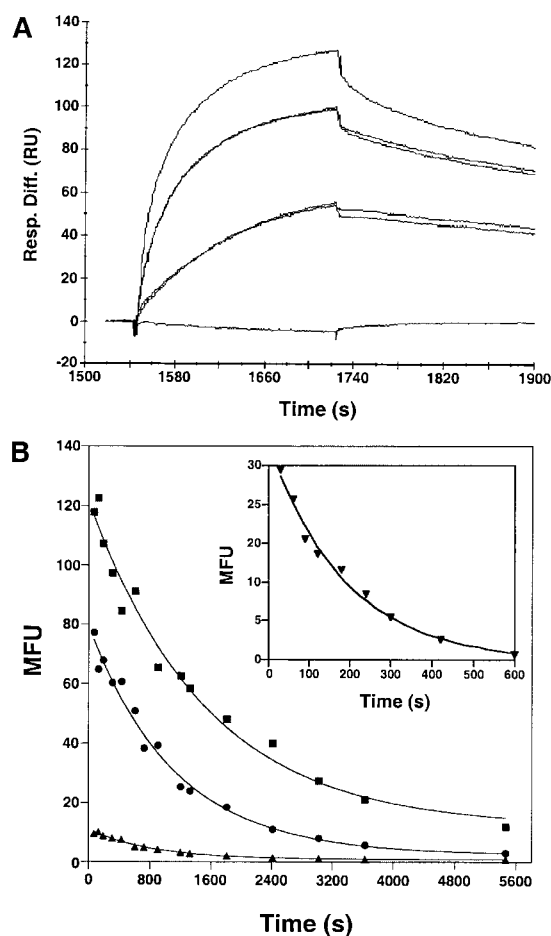


Figure 2. Kinetics of peptide/L^d binding by soluble, high-affinity TCR m6. (A) Representative SPR sensorgrams of soluble m6 TCR binding to QL9/L^d. Experiments were performed by immobilizing QL9/L^d/Ig by capture with rabbit anti–mouse IgG followed by injections of purified m6 TCR (900 nM, 400 nM, 200 nM, PBS blank; duplicate runs shown from top to bottom, respectively). TCR binding to the null peptide, MCMV/L^d/Ig, immobilized on the chip was undetectable (data not shown). (B) Soluble m6 TCR dissociation from four different cell-bound peptide/L^d complexes. Peptide-loaded T2-L^d cells were incubated with full length m6 TCR for 30 min. After washing, cells were resuspended at room temperature in buffer containing excess competing Ab (F23.2 at 300 μg/ml). At various times, cell aliquots were washed and remaining cell bound m6 TCR was detected by flow cytometry with F23.1–biotin followed by SAV:PE. Data points were fit with an exponential equation to determine dissociation rate constants. Representative data is shown for the peptides QL9 (●), QL9-Y5 (■), p2Ca (▲), and QL9-M5 (▼) (inset). The dissociation rate constants (k_d) determined for the m6 TCR and pMHC ligands were: QL9/L^d, 8.5×10^{-4} s⁻¹ ($n = 3$); QL9-Y5/L^d, 5.9×10^{-4} s⁻¹ ($n = 2$); p2Ca/L^d, 1.2×10^{-3} s⁻¹ ($n = 3$); QL9-M5/L^d, 5.0×10^{-3} s⁻¹ ($n = 2$). The $t_{1/2}$ values calculated from these data were: QL9/L^d, 830 s; QL9-Y5/L^d, 1,180 s; p2Ca/L^d, 580 s; QL9-M5/L^d, 140 s. MFU, mean fluorescence units.

positive for F23.2 binding were examined in an IL-2 release assay at different QL9 concentrations. Clones of wt and m6 TCRs that exhibited similar levels of TCR as judged with F23.1 and F23.2 (Fig. 3) and similar levels of IL-2 release were used in the studies presented. However, each of the IL-2 releasing isolates of wt TCR and m6 TCR transfectants showed relative peptide dose responses

Table I. Summary of TCR-pMHC Binding Constants and Peptide Sensitivity of T Cell Transfectants

2C TCR	^a Binding to QL9/L ^d				IL-2 sensitization dose (SD ₅₀) (nM) ^b				
	k _a	k _d	K _D	t _{1/2}	QL9	Y5	E8	M5	p2Ca
	(M ⁻¹ s ⁻¹) × 10 ⁴	(s ⁻¹) × 10 ⁻⁴	nM	s					
wt	0.6	200	3,000	35	0.9	0.3	6	2	2,000
m6 mutant	4.2	4.2	10	1,650	0.003	0.0008	0.8	0.01	40

^aBinding measurements from SPR studies. TCR/pMHC half-lives (t_{1/2}) were calculated using the formula ln 2/k_d. wt 2C TCR data are from reference 12.

^bData from peptide titrations of T2-L^d cells incubated with 58^{-/-} transfectants. Sensitization doses (SD₅₀) were calculated as the peptide concentration that yielded 50% of maximal IL-2 release, by fitting the ascending portions of the IL-2 response curves (Fig. 2). Values reported are the means of two or three independent experiments, each agreeing to within fourfold.

identical to those described below (data not shown). F23.1/F23.2⁺ transfectants were also examined for their ability to bind directly to QL9/L^d/Ig. At the concentration tested (100 nM; above the K_D of m6, but below the K_D of wt), QL9/L^d/Ig binding was detected only with the m6 transfectants (data not shown).

Transfectants were examined in a 30 h IL-2 secretion assay in the presence of various peptides and T2-L^d target cells. As shown in Fig. 4, peptide-specific IL-2 release was observed for both the wt TCR and m6 TCR lines. However, the m6 transfectant showed remarkable sensitivity to the peptides, such that they were stimulated at 10 to 300-fold lower peptide concentrations than the wt transfectants (summarized in Table I). This enhanced sensitivity was exhibited for each QL9 variant and it was reproducible among different lines that were isolated from the wt and m6 transfections (data not shown). Thus, not only were T cells that expressed the high-affinity TCR active, but they were surprisingly more sensitive to peptide levels. This finding suggests that the concept of an affinity or off-rate optimum should be revised, at least to the extent of the affinity of the m6 TCR. It also may indicate that the in vitro engineering of TCRs for higher affinity could prove useful for increas-

ing the activity of T cells against particular pMHC targets, for example in adoptive T cell therapies (26).

The serial triggering model suggests that multiple TCRs on the surface of a T cell can be bound by a single pMHC complex and that this serial binding leads to enhanced signaling. While TCRs on the m6 transfectants are efficiently downregulated by pMHC (see below), the longer off-rates of these interactions would have led to the prediction that fewer serial triggering events could occur compared with the wt TCR transfectants. Despite this prediction, the m6 T cells exhibited rapid downregulation of TCRs and enhanced activity (see below). These results support the notion that serial triggering is itself unnecessary for T cell activity and in fact may be detrimental to it (27–29). What then is the mechanism by which the m6 T cells are able to efficiently recognize and be triggered by pMHC? We propose that the longer lifetimes of TCR-pMHC association allow for enhanced dimerization or clustering of other nonliganded TCR complexes with the liganded TCR on the surface of the T cell. The potential importance of dimerization in T cell activation has been raised previously in regard to the restricted orientation of TCR-pMHC interactions (30), and it is discussed further below.

Of course, there are caveats to this study that will need to be explored further: (a) the findings represent a class I MHC system and it remains to be seen if class II-reactive T cells behave in the same manner; (b) the T cells do not express accessory CD8 molecules, which are known to play an important role in binding and signaling and have been shown to differ in their contribution within the 2C system, depending on whether the ligand is K^b or L^d (31); and (c) the activity measured is that of cytokine release, whereas other functions such as proliferation may differ in their response. Examination of these issues may help address the question of why TCRs have not evolved a process of affinity maturation, similar to that of Abs. In this regard, it is possible that the process of negative selection in vivo might limit the development of high affinity TCRs (32).

A recent study using TCRs from K^b-reactive T cells came to the opposite conclusion based on the analysis of

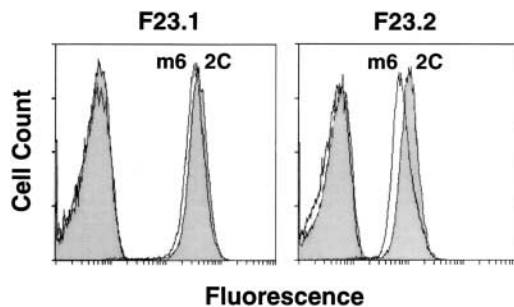


Figure 3. TCR expression by T cell transfectants. T cell hybridomas transfected with wt (2C) or high-affinity mutant (m6) TCRs were stained with biotinylated F23.1 IgG (anti-Vβ8, ~90 nM) or F23.2 IgG (anti-Vβ8.2, ~130 nM) followed by SAV:PE. Cellular fluorescence was measured by flow cytometry. Histograms on the left of each panel are transfectants stained with SAV:PE alone.

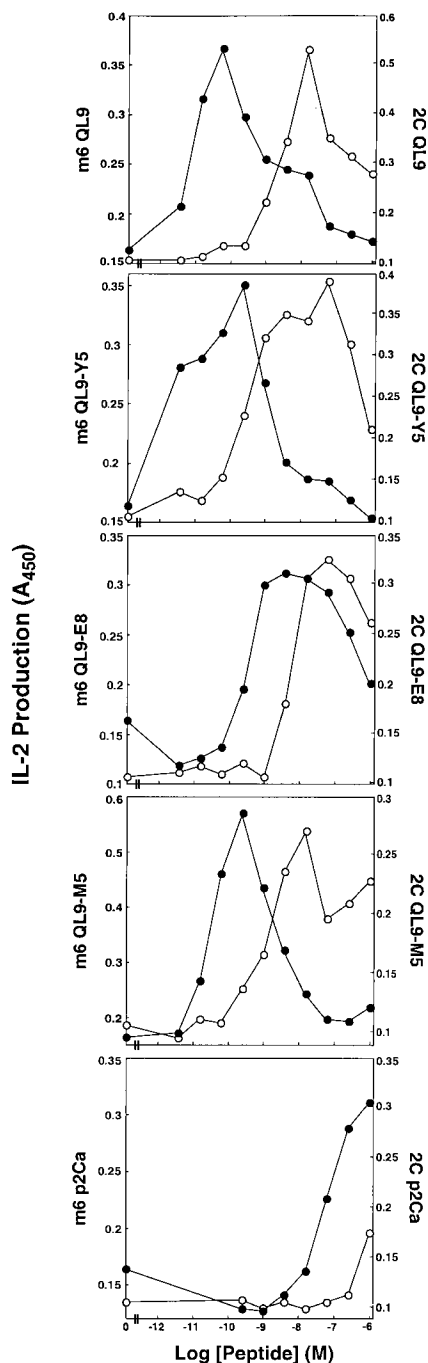


Figure 4. IL-2 release by T cell hybridomas transfected with the wt 2C or high-affinity mutant m6 TCRs. 2C TCR (○) or m6 TCR (●) transfectants were incubated with T2-L^d APCs in the presence of various concentrations of peptides. After 30 h, supernatants were harvested and assayed for IL-2 by ELISA. Assays were done in triplicate and the mean A₄₅₀ value for each concentration is shown. ELISA curves from one representative experiment are shown for each peptide and transfectant. IL-2 standard curves demonstrated that an A₄₅₀ value of 0.3 corresponded to ~1,200 pg/ml IL-2. The null peptide MCMV did not elicit detectable IL-2 at even the highest concentration tested (1 μM).

several TCR mutants (17). In their study, the mutants had several fold higher binding avidity than the wt TCR, but they exhibited reduced biological activities. It is unclear to us what accounts for this discrepancy, especially since the affinities of the m6 TCR for virtually all of the pMHC examined here were orders of magnitude higher than in their system. A possibility, albeit remote, is that CD8 α expressed by the transfectants in their study, affected the signaling through the TCR/CD8 complex.

Effect of Peptide/MHC Stability in T Cell Activation. In the course of studies among different T cell systems, there have been several notable exceptions to the correlate of biological activity and affinity/lifetime of the TCR-pMHC interaction (for a review by Davis, see reference 2). Various hypotheses have been suggested to account for these exceptions, including the possibility that differences in affinity might exist at the temperature used for binding measurements (e.g., 25°C) and that used in the T cell assay (37°C) (24, 33). Other possibilities are that TCRs may undergo conformational changes that impact dimerization and/or signaling or that other T cell accessory molecules, like CD8, could play a role in modulating activity (14, 31, 33, 34). The broad range of affinities among the interactions examined here provides a useful system to reexamine this issue.

The binding of m6 TCR to p2Ca is considerably higher than the binding of m6 TCR to QL9-R5 and QL9-E8 and the dissociation rate of the m6 TCR-p2Ca/L^d interaction is slower than the m6 TCR-QL9-M5/L^d interaction (Fig. 2 B). However, the biological activity of the p2Ca peptide is 2 to 3 orders of magnitude lower than these peptides (Fig. 4, and data not shown). Similarly, the m6 TCR binds much better to p2Ca/L^d than the wt TCR does to QL9/L^d, but the latter stimulates the wt TCR transfectants an order of magnitude more efficiently.

The explanation for these differences almost certainly lies in the stability of these various pMHC complexes. Peptide p2Ca is ~100-fold less stable than QL9 and 300-fold less stable than the QL9-M5 variant in L^d upregulation assays (24). This stability can be demonstrated by examining the amount of appropriate pMHC complex on the T2-L^d cells that remains at the end of the 30 h IL-2 assay. As shown in Fig. 5, p2Ca/L^d complexes are not even detectable at the highest concentration of peptide tested (1 μM), whereas in a shorter assay as used for the original binding studies, these complexes are easily detected (Fig. 1). This contrasts with the QL9-M5 peptide which is detectable at about the same level as p2Ca in a short assay (Fig. 1) but remains easily detectable at the end of the 30 h IL-2 assay (Fig. 5). These results are consistent with the greater persistence of the QL9-M5/L^d complex at lower peptide concentrations (e.g., note the relative levels of QL9-M5/L^d, QL9-Y5/L^d, and QL9/L^d complexes detected at 20 nM peptide in Fig. 5). Identical results were observed for T2-L^d cultures incubated with peptides for 30 h in the absence of T cell transfectants and with anti-L^d Ab used as the detecting agent (data not shown).

TCR binding assays used here and elsewhere typically involve measurements at 25°C and over a time scale of minutes. In addition, peptide–MHC binding assays to measure the affinity of a peptide for MHC product are also performed in a shorter time frame. However, biological assays such as IL-2 release and CTL cytotoxicity assays typically involve several hours to days and are performed at 37°C. Thus, the effects of peptide stability can be greatly magnified in a biological assay. The results with p2Ca are a useful example in this regard. While the comparison of m6–p2Ca/L^d with m6–QL9-R5/L^d or m6–QL9-M5/L^d may represent extremes with regard to stability and TCR affinity, the results do emphasize an important point. Minor, perhaps undetectable, differences in peptide–MHC binding and stability over a short assay period may still yield significant biological effects in long term assays. Thus, we propose that “apparent” exceptions (1, 14–16) to the positive correlation between affinity (or off-rate) and biological activity can be accounted for by pMHC stability. In the 2C system, it seems likely that the 100,000-fold difference in biological activity between dEV8/K^b and SIYR/K^b (35) is due to the greatly reduced stability dEV8/K^b has compared with SIYR/K^b (unpublished data). Recent studies have further emphasized the importance of pMHC stability in eliciting T cells (36) and the impact of minor structural changes in the pMHC on stability (37).

T Cell Inhibitory Effects at High Peptide Concentrations. In experiments performed with wt TCR and m6 TCR transfectants, there was reduced IL-2 release at high concentrations of the most potent peptides (Fig. 4). What could ac-

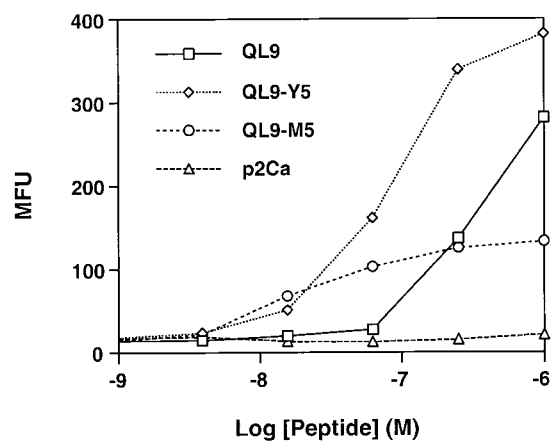


Figure 5. Long-term stability of peptide/L^d complexes. Specific peptide/L^d complexes that remained on the surface of T2-L^d at the end of a 30 h IL-2 assay were quantitated by detection with soluble m6 TCR. T2-L^d cells were incubated with 2C TCR or m6 TCR transfectants in the presence of various concentrations of peptides. After 30 h, cells were harvested and peptide/L^d levels on T2-L^d were measured by staining with soluble m6 TCR followed by F23.1-biotin/SAv:PE and flow cytometry. Only those peptide variants (QL9, QL9-Y5, QL9-M5, and p2Ca) that showed the highest mean fluorescence units (MFU) with m6 (see Fig. 1) are shown. The lower level of signal with QL9-M5, compared with QL9 and QL9-Y5, is due to reduced binding affinity of QL9-M5/L^d for the m6 TCR.

count for this reduction in responsiveness? One possibility is that continued stimulation during the course of the 30-h assay may result in extensive TCR downregulation (9). As it is known that TCR internalization occurs constitutively, in the absence of signaling, the loss of TCRs due to downregulation might yield T cells that are incapable of sustained IL-2 release. Consistent with this possibility, all transfectants that displayed lower IL-2 levels at high concentrations of peptides exhibited extensive loss of TCRs (Fig. 6). T cells incubated with peptides at concentrations that yielded optimal IL-2 release (or less potent peptides such as p2Ca) did not exhibit this extensive TCR downregulation. Thus, the loss of TCRs at high pMHC density could account for reductions in signaling and reduced IL-2 levels. Analysis of the kinetics of the downregulation have shown that the m6 T cell lost over 90% of surface TCR and the wt T cell lost 80% of surface TCR in only 3 h. Thus, despite the high affinity and slow off-rate of the m6 TCR, it undergoes rapid downregulation. The profound, sustained, downregulation of TCRs during the entire course of the assay could cause desensitization of the T cells so that they are incapable of releasing IL-2.

The present data does not allow us to address the current debate on whether nonengaged TCRs might also undergo downregulation (28), for the following reason. We have estimated that at QL9 concentrations above 10 nM there are likely to be ~20,000 QL9/L^d complexes per T2-L^d cell (38). In a 3-h assay there is sufficient time for a single QL9/L^d to undergo multiple rounds of association/dissociation with a number of m6 TCRs (given the lifetime of dissociation

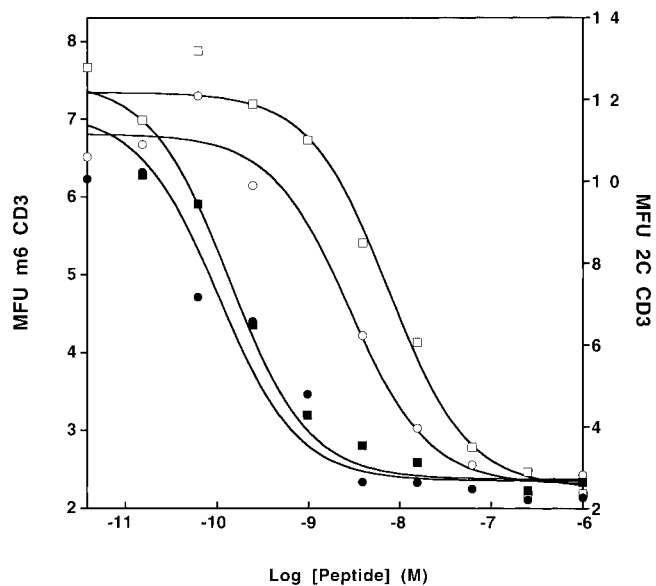


Figure 6. TCR/CD3 downregulation on TCR transfectants. 2C TCR or m6 TCR transfectants were incubated with T2-L^d in the presence of various concentrations of peptides. After 30 h, cells were harvested and TCR levels were measured by flow cytometry with an anti-CD3 Ab (2C11). Representative data from one assay is shown for the 2C wt TCR transfectant incubated with QL9 (□) and QL9-Y5 (○) and the m6 TCR transfectant incubated with QL9 (■) and QL9-Y5 (●).

tion of 1650 s at 25°C) yielding the observed 90% m6 TCR downregulation. Lower QL9 concentrations, where the number of QL9/L^d complexes would be more limiting, yielded minimal downregulation after 3 h incubation (data not shown).

Lower peptide concentrations that yield minimal downregulation nevertheless generate the most productive signaling and IL-2 release. Presumably, there are ligand-specific signals that dictate a TCR will be marked for internal degradation and therefore, not recycled to the surface (29). These signals are likely to differ from the signals initiated by a TCR that lead to IL-2 secretion. Liu et al. (29) have suggested that TCR ligation signals leading to TCR downregulation might involve dissociation of the CD3 ζ chain. In considering the possible differences between signals that lead to reduced TCR recycling and IL-2 secretion, we suggest that the valency state of the TCR-pMHC interaction may provide an answer. Our thoughts in this regard were prompted by the remarkable similarity in the "Gaussian" distribution of peptide-induced IL-2 release (Fig. 4) and the results of recent experiments that examined the cross-linking of TCRs by bivalent pMHC ligands (39). In the latter studies, reduced TCR dimerization was observed at high concentrations of the bivalent pMHC, as monovalent interactions predominate at these concentrations. In the system described here, monovalent TCR interactions could also predominate at high peptide concentrations, leading to efficient downregulation without activation. At high pMHC levels, each TCR could be "immobilized" in the T cell membrane, preventing association with neighboring TCR complexes. In contrast, TCR dimerization might be achieved at lower concentrations because a ligated TCR-pMHC complex can associate with a membrane mobile, nonligated TCR, leading to productive IL-2 release. The frequency of TCR dimerization would be dictated by the stability of the pMHC and the affinity of the TCR-pMHC. The observation that membrane mobility is very important in TCR-pMHC binding and T cell function has been well documented (39-41) and a number of studies have suggested that the α/β TCR may exist as a dimer or higher order oligomer (33, 42, 43). This model would also allow for the enhanced signaling effects of CD8 accessory molecule that could facilitate the formation of dimeric TCR complexes through interactions with MHC (31, 34). Interaction of a CD8 molecule in a nonligated TCR complex with a pMHC molecule that is engaged by a neighboring TCR could greatly facilitate the formation of TCR dimers. Obviously, these possibilities will need to be examined further and the TCR transfection system described here should facilitate these studies.

Concluding Remarks. It has been suggested that the relatively low affinities and fast off-rates of TCR-agonist pMHC interactions represent an optimal window for eliciting T cell activity. This hypothesis has received conceptual support from the serial triggering model, which proposes that longer lifetimes of TCR-pMHC engagement would lead to fewer interactions of a single pMHC with different TCRs. The early observations that TCRs lack so-

matic mutation and consequent affinity maturation (at least not to the same level as Abs, with nanomolar affinities), also supported the notion that T cells are not advantaged by increasing the intrinsic binding affinities of their TCRs (for a review, see reference 44). In apparent contradiction to these predictions, we show here that T cells expressing a high affinity TCR, produced in vitro by directed evolution, are exquisitely sensitive to low pMHC levels and that they are fully capable of activation. We propose that the improved T cell activity associated with longer lifetimes of TCR engagement is inconsistent with the notion that serial triggering is required for signaling. We believe the findings also support the concept that TCR dimerization is important in T cell activity and that it would be enhanced with higher affinity TCRs.

The results imply that there must be other explanations for why T cells have not evolved a process that yields high affinity TCR. In fact, the relatively low affinities of TCR-pMHC complexes are typical of most protein-protein interactions involved in cell to cell contact (45). It is possible that such affinities are necessary to maintain the full spectrum of functions (e.g., migration, differentiation, proliferation, etc.) that hematopoietic cells undergo. For T cells, perhaps negative selection eliminates TCRs that might have exhibited high affinity against a foreign peptide because they also cross-react with self-peptides. Another possibility is that other T cell activities, notably proliferation, might be compromised by the presence of higher affinity TCR. It will be important to examine this issue by introducing our mutant TCRs into CTL lines or transgenic mice.

A further finding of the present study is that pMHC stability plays a dominant role in T cell activity. Although many studies have shown the importance of pMHC stability, the examples shown here illustrate how pMHC stability can dominate the TCR-pMHC binding interaction. Thus, we propose that apparent discrepancies between TCR affinity and T cell activity observed in some prior studies can be accounted for by full consideration of the binding affinity of the peptide for MHC and the stability of the pMHC complex at 37°C.

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