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Interaction of Streptavidin-Based Peptide–MHC Oligomers (Tetramers) with Cell-Surface TCRs

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The binding of oligomeric peptide–MHC (pMHC) complexes to cell surface TCR can be considered to approximate TCR–pMHC interactions at cell-cell interfaces. In this study, we analyzed the equilibrium binding of streptavidin-based pMHC oligomers (tetramers) and their dissociation kinetics from CD8pos T cells from 2C-TCR transgenic mice and from T cell hybridomas that expressed the 2C TCR or a high-affinity mutant (m33) of this TCR. Our results show that the tetramers did not come close to saturating cell-surface TCR (binding only 10–30% of cell-surface receptors), as is generally assumed in deriving affinity values (Kd), in part because of dissociative losses from tetramer-stained cells. Guided by a kinetic model, the oligomer dissociation rate and equilibrium constants were seen to depend not only on monovalent association and dissociation rates (koff and kon), but also on a multivalent association rate (μ) and TCR cell-surface density. Our results suggest that dissociation rates could account for the recently described surprisingly high frequency of tetramer-negative, functionally competent T cells in some T cell responses. The Journal of Immunology, 2011, 187: 6281–6290.

Because Abs and many Ags are soluble, it has been possible to study their interactions with a variety of methods under conditions that are physiological or nearly so. For TCRs and their peptide–MHC (pMHC) ligands, however, their natural state as integral membrane proteins on T cells and APCs limits the options for analyzing their interactions. Considerable insights have been gleaned from responses of T cells to pMHC displayed at various levels on other cells (target cells or APCs). The responses are informative particularly when correlated with measurements of equilibrium constants and reaction rates, but the latter are most often determined with rTCR and MHC molecules in the absence of CD8 and CD4 coreceptors. Because these coreceptors have a pronounced impact on the cellular responses, efforts have been made to study the binding of soluble pMHC complexes to TCR on intact CD8pos T cells (1). As monomers, these complexes are of limited use because they dissociate too rapidly from TCR (2, 3). However, pMHC oligomers bind more stably. Hence, they are widely used to identify T cells with cognate TCR and also, though much less widely, to determine TCR–pMHC affinities and reaction rates. The oligomeric forms include IgG dimers (4) and pentamers (ProImmune, Oxford, U.K.), but most often, as in the current study, streptavidin (SA)-linked pMHC oligomers, called tetramers (5–7), as originally introduced by Altman et al. (8).

It has been generally accepted that the proportion of T cells that are stained by chromophore-labeled tetramers accurately measures the frequency of T cells that express the corresponding (cognate) TCRs. There are reports, however, of CD8pos T cells that respond specifically to pMHC on target cells, yet are not stained by the same pMHC as tetramers (9–12). Recently, a surprisingly high frequency of CD4pos T cells that are similarly tetramer-negative but functionally competent has been described in responses to infection by lymphocytic choriomeningitis virus and especially to a self-antigen [myelin oligodendrocyte glycoprotein (13)].

Studies have shown that the intensity of tetramer staining of T cells generally correlates with monovalent TCR–pMHC affinity and several other variables, including the density of TCR on T cells, lipid membrane organization, and differentiation status of the cells [activated versus naive T cells (12, 14–18)]. Estimates of the multivalent affinity (avidity) of cell surface TCR for tetramers are taken as the concentration of free tetramer at half-maximal binding of tetramer to cells, a determination that assumes saturation of surface TCR by bound tetramers at high free tetramer concentration. This assumption was questioned by a recent study (19), in which a panel of TCR that differed widely in affinity for the same pMHC was expressed in hybridomas that were stained with that pMHC in tetrameric form. Although the TCR levels were expressed at the same levels in all hybridomas, the maximal levels of tetramer staining varied considerably, raising the possibility that cell surface TCR were not saturated in any of the cells tested.

To evaluate this possibility, we analyzed in this study the equilibrium binding of pMHC tetramers and their dissociation kinetics from CD8pos T cells from 2C TCR-transgenic mice and transduced T cell hybridomas that expressed the 2C TCR (20) or an engineered high-affinity mutant [m33 (21)] of this TCR. The analyses were based on a kinetic model of multimeric pMHC binding to cell surface TCR. The results establish that tetramers...
did not come close to saturating cell-surface TCR, in part because of their dissociation when tetramer-stained cells are washed. Besides the intrinsic (monovalent) association and dissociation rates, critical determinants of tetramer dissociation are the multivalent association rate (μ) and the two-dimensional (2D) concentration (density) of cell-surface TCR. The rapidity of dissociation of tetramers from some TCR can account for the frequency of tetramer-negative, functionally competent T cells.

Materials and Methods

Peptides, Abs, and cells

SIY (SIYRYGL) and OVA (SIINFEKL) 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, and masses were confirmed by MALDI. Peptide quantification by amino acid analysis was performed at the Molecular Structure Facility, University of California, Davis (Davis, CA). Fluorescein-labeled mAbs (F23.1, H57-597, 145-2C11, 53-6.7, and 53-5.8) and SA were purchased from BD Pharmingen (San Jose, CA). The 1B2 anti-2C TCR and B.8.24.3 anti-K\(^{\alpha}\) mAbs were purified from hybridoma supernatant using protein G beads. 1B2 was labeled with FITC, and purified to remove excess FITC. For each fluorescein-labeled protein, the protein concentration and number of fluorescein molecules per protein molecule was determined by comparing the ratio of UV-Vis absorbance at 495 nm and 280 nm (PBS [pH 7.4]), using a molar extinction coefficient of 280 nm for fluorescein. The number of fluorescein molecules was determined for model tetrameric and dimeric complexes as described (19). The refolded protein was purified by binding to Ni-NTA agarose beads (Qiagen, Valencia, CA), followed by elution in 500 mM imidazole, followed by size exclusion chromatography over a Superdex 200 gel filtration column (GE Healthcare, Piscataway, NJ).

Protein expression and preparation

Single chain V\(\beta\)-linker-V\(\alpha\) TCR (scTCR) were expressed as inclusion bodies in BL21(DE3) Escherichia coli (Stratagene, La Jolla, CA). The stable single chain 2C TCR that includes an interdomain flexible linker (22) has been previously shown to maintain binding specificity for all ligands tested, and binding measured by surface plasmon resonance (SPR) has shown identical binding affinity and kinetics for the scTCR and full-length, soluble TCR without a linker (23, 24). Proteins were solubilized in urea and refolded as previously described (19). The refolded protein was purified by binding to Ni-NTA agarose beads (Qiagen, Valencia, CA), followed by elution in 500 mM imidazole, followed by size exclusion chromatography over a Superdex 200 gel filtration column (GE Healthcare, Piscataway, NJ).

H2-K\(^{\alpha}\) H chain containing a C-terminal biotinylation signal peptide and human β2 microglobulin L chains were expressed separately in E. coli. H2-K\(^{\alpha}\) H chain was biotinylated in vivo by coinduction of biotin ligase, so that the H chain carried a biotin tag (25). Both chains were expressed as inclusion bodies, solubilized in urea and refolded together in vitro in the presence of excess SIY or OVA peptide (26). Folded complexes were purified by anion exchange chromatography using HiTrap Q columns (GE Healthcare, Piscataway, NJ) and size exclusion chromatography. For incorporation of SIY\(^{\alpha}\) into SA-oligomers, precharacterized, calibrated, fluorescein-labeled SA was added stepwise to the biotinylated SIY-K\(^{\alpha}\) complexes in small aliquots on ice over 20 min to various final molar ratios. Characterization of the resulting oligomer complex distribution was performed by SDS-PAGE.

Binding measurements of soluble receptors at 10°C

Kinetic and equilibrium binding data were obtained by SPR using a BIACore 3000 (Biacore Life Sciences, GE Healthcare, Piscataway, NJ) precooled to 10°C, with 0.02% (v/v) Tween and 0.005% (v/v) Tween-20 (pH 7.4). Binding of scTCRs to the null complex OVA\(^{\alpha}\) was subtracted from TCR binding to SIY\(^{\alpha}\) to correct for bulk shift and any nonspecific binding. On-rates, off-rates, and kinetic-based K\(_{\text{D}}\) analyses were performed using BIACalculator 3.0 software (Biacore Life Sciences).

Oligomer binding and dissociation experiments

To perform steady-state oligomer binding titrations, 58\(#^\text{+}\) cells transduced with TCR genes (2C, m33, or other mutants) or T cells purified from 2C transgenic or C57BL/6 mouse splenocytes were incubated with various concentrations of fluorescein-labeled SA-SIY\(^{\alpha}\)-kickers in FACS buffer (1% BSA in PBS with 0.02% sodium azide) on ice for at least 2 h in the dark. After an 8 min wash in cold (~10°C) FACS buffer, cells were resuspended in cold FACS buffer and analyzed for bound fluorescent tetramers by flow cytometry. Fluorescence levels of the parental 58\(#^\text{+}\) cell line (control) were subtracted as background from the TCR transfected hybridoma values at the same staining concentration.

Oligomer dissociation experiments were performed as described previously (19, 26–28). Briefly, 58\(#^\text{+}\) cells transduced with mutant TCR chains or 2C TCR transgenic T cells purified from mouse splenocytes were stained with 293 nM (or 5.85 \(\mu\)M for 2C TCR hybridomas without CD8\(\beta\)) SA-linked SIY\(^{\alpha}\)-TCR tetramers on ice for 2 h. Cells were washed three times in cold 25°C FACS dissociation buffer containing 0.1% azide, 100 \(\mu\)M cytochalasin D, and 200 \(\mu\)g/ml K\(^{\alpha}\)-blocking Ab (B.8.24.3, to prevent rebinding) 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\(#^\text{+}\) cell line or for C57BL/6 cells. Data from dissociation experiments were fit by an equation describing a first-order exponential decay.

Quantification of cell surface-bound Abs and oligomers

Cells were stained at 4°C in the dark with saturating amounts of calibrated fluorescein-labeled Abs (determined by titration), or the indicated levels of SIY\(^{\alpha}\) oligomer made with calibrated fluorescein-labeled SA, for at least 2 h. The cells were then washed for 8 min at 4°C in a large excess of FACS buffer, and resuspended immediately prior to analysis by flow cytometry (FACS Canto, BD Biosciences, San Jose, CA). Quantification of molecules per cell was derived from flow cytometry experiments in which specific fluorescence was analyzed in relation to calibrated fluorescein beads (Spherotech, Lake Forest, IL and Bangs Labs, Fishers, IN, Supplemental Figs. 1, 2). Fluorescent beads were analyzed at the same cytometer settings as the stained cells, and measured fluorescence values were used to convert corrected cell-bound fluorescence to numbers of cell-bound fluorescein-molecule equivalents. These latter values were used to calculate the number of cell-bound Ab molecules or SA-oligomers.

Model

To describe the behavior of SA-linked oligomers binding to and dissociating from T cells, we developed and applied a quantitative model. As the oligomers used in this study have on average three pMHC complexes per SA molecule (Fig. 1A, below), our model assumes that an SA-oligomer can bind to one, two, or three cell-surface TCR molecules; these various bound states are described as \(L = T\), \(L = T\), and \(L = T\), where \(L\) refers to oligomer (ligand) and \(T\) to TCR. As discussed below (in Results) and shown in detail in Appendix A: Model Equations, a bound oligomer’s association rate is determined by \(k_{\text{on}}\), while its intrinsic dissociation rate of the pMHC–TCR bond, as measured, for example, by SPR and \(\mu\) (a multivalent \(k_{\text{on}}\) with units of \(\text{s}^{-1}\)), is related to the intrinsic [univalent] pMHC–TCR association rate, \(k_{\text{on}}\) with units of \(\text{M}^{-1}\text{s}^{-1}\), and an effective concentration of surface \(T\), as discussed below (in Results).

At equilibrium, the oligomer association and dissociation rates are equal, as described by: \(k_{\text{on}}\) \(T_{\text{free}}\) \(L_{\text{bound}}\) = \(k_{\text{off}}\) \(T_{\text{bound}}\) \(L_{\text{free}}\), where \(k_{\text{on}}\) is on-rate for the monomeric pMHC–TCR interaction, as measured for instance by SPR, modified by the number of pMHC per SA molecule. If \(\theta\) is the fraction of occupied receptors, \(T_{\text{free}}\) = \((1 - \theta)\) \(T_{\text{total}}\), and \(L_{\text{bound}}\) = \(\theta L_{\text{total}}\), we can express the ratio of bound to free receptors as Eq. 5, which resembles the Scatchard equation (29–31), then follows. Detailed derivation of the equilibrium equations (including Eq. 5) can be found in Appendix A: Model Equations. In describing these multivalent binding events, we assume that a pseudo-equilibrium between the T cell-bound states of an oligomer is reached rapidly compared with the overall association or dissociation of an oligomer from the T cell. This assumption is justified where \(\mu\) > \(k_{\text{off}}\) as is the case
for the TCR–pMHC interactions described in this study (µ∼10-40-fold larger than \( k_{off} \)). In addition, our interactions are in the range where dissociation data can be described by an exponential decay curve; a lack of rapid interconversion of bound states would result in a different shape to the dissociation data (32). In a range where this assumption is not valid, the amount of bound oligomer over time could be determined by numerically solving the set of differential equations defining the interactions in Appendix A: Model Equations (Eq. A1–A3). Moreover, with the assumption \( \mu > > k_{off}, k_{eff} \), can be obtained from Eq. 2 in the text, the derivation of which can be found in Appendix A: Model Equations.

In this model, we also assume no positive or negative cooperativity of binding, which could affect on-rates or off-rates beyond the statistical factors we have applied to \( \mu \) and \( k_{off} \). Although this assumption may not be fully accurate, there is no clear way to account for such effects. Based on the successful application of this model to our data, we suspect that any cooperativity, if present, does not affect our overall conclusions.

Results
Characterization of pMHC–SA-linked oligomers (tetramers)

The complexes formed by SA with biotinylated MHC, usually called tetramers, vary in the number of biotinylated MHC molecules per SA molecule (33, 34). Fig. 1A shows the distribution of oligomers when biotinylated class I MHC and SA were combined at different molar ratios. Based on these results, the preparations used for subsequent work (below) were, assembled with a 16:1 molar ratio of SIY/K\( ^b \)-SA; they consisted of ~40% trimer, and 20% each tetramer, dimer, and monomer bound to SA. Because of the size distribution (Fig. 1A), we refer to them subsequently as SA-oligomers, or simply oligomers, rather than as tetramers.

Analysis of steady-state binding of pMHC oligomers to T cells

To relate oligomer binding to TCR affinity, CD8 contribution, and cell surface levels of TCR and CD8, we first measured the steady-state binding of SIY/K\( ^b \) oligomers to T cell hybridomas that expressed the 2C TCR (20) or an engineered, high-affinity mutant [m33 (21)] of this TCR (Fig. 1B), and also to naive CD8\(^b\) 2C T cells freshly isolated from spleens of 2C TCR transgenic mice (Fig. 1C). CD8\( ^a \) and CD8\( ^b \) were coexpressed in some of the hybridomas. After the cells were incubated on ice for 2 h with oligomers at various concentrations, they were dispersed in a large volume of cold buffer, centrifuged, and the pelleted cells resuspended immediately before analysis by flow cytometry. The cell-bound oligomers increased with increasing free oligomer concentration to a maximum level and reached a plateau, or declined slightly, at the highest concentrations (Fig. 1B, 1C). The treatment used in this study is typical of oligomer staining protocols where binding is allowed to reach steady-state, and the cells are washed prior to analysis by flow cytometry. Although these data may be fit by a sigmoidal curve similar to equilibrium binding data giving a 50% maximum value (\( K_{D,olig} \)) that can be compared from cell population to cell population, this does not precisely correspond to a true equilibrium binding constant (see below). To obtain a more representative equilibrium on a cell surface, techniques such as spinning cells through oil rapidly to remove unbound ligands (1) or avoiding a wash step entirely (15) have been employed previously.

As shown in Fig. 1B, SIY/K\( ^b \) oligomers bound equally well to 1) hybridoma cells (58\(^2 \)) that expressed CD8 but not 2C TCR and 2) hybridoma cells that expressed the 2C TCR but not CD8. This finding is consistent with the similar binding constants found for the binding of CD8\( ^a \) \( \beta \) to peptide-K\( ^b \) [\( K_D = 38 \mu M \)] and 2C TCR to SIY-K\( ^b \) [\( K_D = 30 \mu M \)] (19, 23, 24).

The TCR affinity for the oligomers is generally taken to be the free oligomer concentration when the amount of bound oligomers is half of the plateau or maximal level (\( \Gamma_{D,olig} \)). The ratio between \( \Gamma_{D,olig} \) and the equilibrium constant (\( K_D \)) for monovalent binding of the same TCR to the same pMHC as monomer (\( K_{D,olig}/K_D \), called the enhancement factor) was previously seen to be more pronounced for low-affinity than high-affinity TCR–pMHC interactions (19). It was thus not surprising that for m33, the very high-affinity TCR (19), the oligomer concentration that resulted in half-maximal binding was about the same with CD8\(^a\) and CD8\(^b\) hybridomas (Fig. 1B). However, for the much lower affinity wild-
type 2C TCR, the half-maximal concentrations with CD8neg and CD8pos hybridomas, 2.8 nM and 4.8 nM, respectively, were surprisingly also similar (Fig. 1B); the small difference implied that the coexpressed CD8 had, if anything, a negative effect on this TCR’s affinity for pMHC. Among the questions raised by these findings is whether the SA-oligomers can engage all cell-surface TCR and measure TCR affinity for pMHC.

**Do oligomers at high concentration saturate cell-surface TCRs?**

To determine if saturation is approached, we measured the number of surface TCR molecules per cell, using fluorescein-labeled mAbs to various TCR domains (Cβ and Vβ8) and to the TCR-associated protein, CD3; we also used the fluorescein-labeled clonotypic Ab 1B2, which is specific for the 2C TCR (but does not bind to the 2C mutant m33). The results are shown in Fig. 2A, 2B, and summarized in Table I. As noted in the legend to Table I, the number of surface TCR molecules per cell was estimated to be twice the number of Ab molecules bound (to account for Ab binding bivalently). The number of TCR may be slightly lower, as some anti-TCR Ab molecules may be bound monovalently. Studies have shown that although the TCR complex can assemble as a monomer (36), the TCR may exist on the cell surface, at least in part, as a dimer (37–39) or oligomer (40, 41) and may preferentially bind Abs bivalently (39). It is, of course, possible that not all the TCRs measured by Ab quantification are conformationally able to bind pMHC at any given time.

As the oligomers are trimeric on average (Fig. 1A), and are thought to behave functionally as trimers, the number of TCR engaged by bound oligomers was taken to be (at most) three times the maximum number of bound oligomers in the titrations shown in Fig. 1B, 1C. From the ratio of oligomer-engaged TCR to the total number of TCR it appeared that not >10–30% of cell surface TCR was maximally engaged by bound oligomers.

**Dissociation kinetics of cell-bound tetramers**

One possible reason for the apparent failure to engage more cell surface TCR is that bound oligomers are lost when cells are washed prior to flow cytometry. To evaluate this possibility, we examined the dissociation of SIY/Kb oligomers from oligomer-stained cells (Fig. 3A, 3B). The steps involved in dissociation can be represented by:

**FIGURE 2.** Detection of T cell receptor and CD8 epitopes on T cell hybridomas and transgenics. Quantification of molecules per cell is derived from flow cytometry staining data using calibrated, fluorescein-labeled Abs and comparing to two independent batches of fluorescein-labeled beads. A–D, Staining of cell-surface epitopes including (A, C) TCR Cβ (clone H57-597), TCR Vβ8 (clone F23.1), CD3ε (clone 145-2C11), the 2C TCR specifically (clone 1B2), and (B, D) CD8α (clone 53-6.7) and CD8β (clone 53-5.8) on (A, B) T cell hybridomas or (C, D) mouse T cells. Error bars represent the SD for at least two and up to eight repetitions for each individual measurement. E and F, Contrast images showing the size differences between (E) mouse T cells (2C TCR transgenic) and (F) T cell hybridomas (m33 TCR).
where \( T \) refers to available TCR sites on the cell surface, \( L = T \) is the number of oligomers bound by three pMHC complexes, \( L = T \) is the number bound by two pMHCs, and \( L - T \) is the number bound by one pMHC. Dissociation of \( L-T \) loses oligomers into solution (\( L_{\text{sol}} \)). The off-rate constants for dissociation (\( k_{\text{off}} \)) are considered to be the same as measured by SPR with monomeric pMHC except for the statistical factors shown in Eq. 1. However, the on-rates will differ by more than these statistical factors. When a TCR monomer binds to immobilized pMHC complexes (as in an SPR experiment), it gives up the entropic freedom of sampling a multitude of configurations in solution and orients itself in the correct configuration. This entropic penalty is contained in the free energy barrier that is included in the measured value of the on-rate constant (\( k_{\text{on}} \)). The same entropic factors (with minor differences due to potential steric stericities) are also relevant when the first pMHC of an oligomer binds, but after one of an oligomer’s pMHC binds to a cell, the binding rate of the other pMHC of that oligomer is determined by rather different entropic changes. Therefore, the effective on-rate for the binding of the second and third pMHC, designated \( \mu \), will differ from that obtained from SPR measurements with monomeric pMHC by more than statistical factors. By assuming that interconversion among bound states of the oligomer (single, double, or triple-bonded) is relatively rapid, it can be shown (see Appendix A: Model Equations) that

\[
L = T \left( 1 - \frac{k_{\text{off}}}{k_{\text{on}}} \right) = T \left( 1 - \frac{2k_{\text{off}}}{2k_{\text{on}}} \right) - T \frac{k_{\text{off}}}{k_{\text{on}}} L_{\text{sol}},
\]

and

\[
L_{\text{bound,d}} = \exp \left[ -k_{\text{off}} t \right] = \exp \left[ - \frac{k_{\text{off}}}{3k_{\text{off}} + 3k_{\text{off}} \mu + \mu^2} \right] \approx \exp \left[ - \frac{k_{\text{off}}^3}{\mu^2} t \right],
\]

where \( L_{\text{bound,d}} \) is the amount of bound oligomer at a particular time (\( t \)), and \( L_{\text{bound,d}} \) is the amount bound initially.

As seen in Figs. 3A, 3B, the dissociation rates from each of the hybridomas and T cells could indeed be fitted by a single-value exponential decay constant \( (k_{\text{off}})^j \), which is equivalent to \( (3k_{\text{off}}^2 + 3k_{\text{off}} \mu + \mu^2)^j \) in Eq. 2. This suggests that we are in a regime where the assumption of a rapid interconversion of bound states is valid, as has been assumed previously (32).

From the experimentally determined \( k_{\text{off}}^{\text{eff}} \) values (Fig. 3), the amounts of oligomer lost during the \( \sim 8 \) min spent in washing oligomer-stained cells (see Materials and Methods) can be estimated. Correction for these losses still indicated that saturation was far from having been achieved before washing cells, especially for the freshly isolated CD8\(^{\text{pos}}\) splenic T cells. These corrections were based initially on dissociation rates measured at 25°C (Figs. 3A, 3B). But, as the cells were washed in the cold (\( \sim 10^\circ\text{C} \)), where longer \( t_{1/2} \) values are expected, the actual losses were likely even smaller. To find out how much smaller, we took advantage of the intrinsic (monovalent) on- and off-rates for the binding of the SIY/K\(^\text{b}\) complex by the 2C and m33 TCR measured by SPR at 25°C and 10°C (Ref. 19 and Table III). Based upon the following argument, these intrinsic rates could be used, together with the \( k_{\text{off}}^{\text{eff}} \) measured at 25°C, to estimate \( k_{\text{off}}^{\text{eff}} \) at 10°C.

As is discussed in Appendix A: Model Equations, the difference in dimensions of \( \mu \) (measured in s\(^{-1}\)) and intrinsic monovalent on-rates, \( k_{\text{on}} \) (which are measured for reactants in solution and expressed in M\(^{-1}\)s\(^{-1}\)) arises because \( \mu \) already incorporates the TCR concentration in the form of the cell-surface density in the vicinity of the bound oligomer; i.e., \( \mu = k_{\text{on}} \times \text{local surface density of TCR} \), where \( k_{\text{on}} \) is essentially the monovalent \( k_{\text{on}} \) as measured by SPR (see below). On the assumption that the local and overall cell-surface TCR density for a given T cell is the same at 25°C and 10°C, it follows that

\[
\mu_{25^\circ\text{C}} = \frac{k_{\text{on},10^\circ\text{C}}}{k_{\text{on},25^\circ\text{C}}},
\]

Values for \( k_{\text{on}} \) at the two temperatures are shown in Table III for m33 and several other engineered mutants of the 2C TCR. From these values, \( k_{\text{off}}^{\text{eff}} \) at 10°C can be obtained by using the relationships outlined in the mathematical model. The correction for losses during an 8 min wash at \( \sim 10^\circ\text{C} \) indicate that before oligomer-stained cells were washed only \( \sim 15\% \) of the TCR molecules on splenic CD8\(^{\text{pos}}\) T cells and \( \sim 40\% \) of those on the T cell hybridomas were engaged by the SA-oligomers at the highest oligomer concentrations tested (Fig. 1B, 1C, Table II).

The extent to which bound oligomers are lost by dissociation from stained cells varies with the decay constant (\( k_{\text{off},\text{app}} \)) and the time spent preparing cells for flow cytometry (Fig. 3C). Because \( k_{\text{off}}^{\text{eff}} \) and intrinsic TCR–pMHC affinity (\( K_{\text{d}} \)) are correlated (Table III) (12, 16, 18, 19, 26), these losses are negligible for the engi-
neered high affinity TCR. But for lower affinity TCR, such as the 2C TCR with the Y48 or S51/Y48 mutations, ~80–90% of bound oligomers would be lost under conditions that are commonly used to prepare oligomer-stained cells for flow cytometry.

**CD8 effect on TCR–pMHC binding**

From Fig. 3, it is evident that the presence of CD8αβ on the cells led to slower oligomer dissociation. For the wild-type 2C TCR on the CD8<sup>neg</sup> hybridoma, dissociation was so fast that the rate could hardly be measured. However, with CD8<sup>pos</sup> hybridoma that expressed the same TCR at about the same level, t<sub>1/2,app</sub> increased to >2 min (Fig. 3A and inset). For hybridomas that expressed the high-affinity m33 TCR, the oligomer dissociation t<sub>1/2</sub> increased from 5.9 min on CD8<sup>neg</sup> cells to 8.5 min on CD8<sup>pos</sup> cells (Fig. 3B).

To compare T cell hybridomas with T cells freshly isolated from mice, CD8<sup>pos</sup> T cells from Rag<sup>-/-</sup> 2C TCR transgenic splenocytes were examined side-by-side with CD8<sup>neg</sup> m33 TCR<sup>+</sup> hybridomas (Fig. 3B). Interestingly, the oligomer off-rate was nearly identical for the two cell types, despite marked differences in TCR affinities, TCR densities, and CD8 expression.

Why do oligomers dissociate more slowly from CD8<sup>pos</sup> 2C T cells than from CD8<sup>neg</sup> hybridomas that express the same TCR (t<sub>1/2,app</sub> = 5.85 and 2.1 min, respectively) (Fig. 3A, 3B)? The answer likely lies in the much greater number of TCR per cell on the CD8<sup>pos</sup> T cells (~56,000/cell on the CD8<sup>pos</sup> T cells versus ~25,000/cell on the CD8<sup>neg</sup> hybridoma cells, Fig. 2C, 2D, Table I). Moreover, the splenic CD8<sup>pos</sup> T cell diameter is about one-third that of hybridomas (Fig. 2E, 2F). These differences mean that the cell-surface TCR density is on average ~20-fold greater on the splenic T cells than on the hybridomas (188/μm<sup>2</sup> versus 8/μm<sup>2</sup>), resulting in the larger multivalent on-rate (μ) and smaller k<sub>off</sub> (larger t<sub>1/2,app</sub>) on the T cells. This average density found on normal and transgenic T cells is consistent with what has been observed previously (42). Precise measurements of density are difficult, however, as the membranes of lymphocytes are not simple spheres (43), and local variations in TCR density are expected (reviewed in Ref. 44), which could influence binding properties. Because the intrinsic k<sub>off</sub> rate is expected to be the same from the 2C TCR, whether expressed by splenic T cells or the transduced hybridomas, the greater stability of the SA-oligomers on the splenic T cells can be attributed to the faster on-rate (μ) on the T cells.

### Oligomer equilibrium binding constants (K<sub>D,olig</sub>)

Although cell-bound pMHC oligomers do not saturate cognate cell-surface TCR, the titration curves in Fig. 1B, 1C indicate that they saturate a subset of these TCR (19). For this subset, the processes that occur on oligomer binding at steady state can be represented as:

\[
L_{sol} + T \xrightleftharpoons{k_{on}} L - T \xrightleftharpoons{2k_{off}} L = T \xrightarrow{\mu}{3k_{off}} L = T, \tag{4}
\]

where all of the terms are defined as in Eq. 1 except for k'<sub>on</sub>, which refers to the on-rate for the first pMHC of the oligomer to bind. We take k'<sub>on</sub> to be the monomeric pMHC–TCR on-rate (measured for example by SPR), except for a statistical factor that takes into account the number of pMHC per oligomer (three in the present case). The binding data can be treated according to the Langmuir adsorption isotherm to obtain an expression for the fraction of cell-surface sites (i.e., TCR) occupied by the pMHC oligomers as a function of the free oligomer concentration. Accordingly,

\[
0 \leq \frac{\theta}{1 - \theta} = \frac{k_{on} L_{sol}(3k_{off}^2 + 3k_{off}\mu + \mu^2)}{k_{off}^3} = k_{on} L_{sol} k_{off}^3 \mu,
\]

where L<sub>sol</sub> is the concentration of oligomers in solution, and θ is the fraction of sites occupied by oligomers (singly, doubly, or triply bound). In deriving Eq. 5 (see Appendix A: Model Equations), we have assumed that the concentration of oligomers in solution is essentially equal to the initial oligomer concentration and that this approximation is valid when the latter quantity is relatively large.
From the titration shown in Fig. 1C, the apparent affinity \((K_{D,\text{olig}})\) of the 2C TCR on splenic CD8\(^{\alpha\alpha}\) T cells for SA-SIY/K\(^{b}\) oligomers is ~1 nM, as determined by the free oligomer concentration at 50% maximum bound. Comparisons of \(K_{D,\text{olig}}\) to monovalent ligand binding to TCR (e.g., the \(K_{D}/K_{D,\text{olig}}\) ratio) are useful to demonstrate the benefits of multivalent ligand binding to TCR (19). The fraction amounted to 15% of the TCR on CD8\(^{\alpha\alpha}\) splenic T cells from 2C TCR transgenic mice and ~40% on a TCR-transduced hybridoma line. These less-than-saturating levels arise in part from losses of bound oligomers when oligomer-stained cells are washed. The extent of these losses depends on the dissociation or decay constant, \(k_{\text{off,olig}}\), which is related to TCR–pMHC affinity (Fig. 3C and Eq. 2). For T cells for which TCR binds oligomers weakly (large \(k_{\text{off,olig}}\)), nearly all bound oligomers may be lost by dissociation when cells are subjected to commonplace washing conditions. This effect could account for reports of tetramer-negative functionally competent T cells (9–12). Losses of bound oligomers by dissociation from low-affinity TCR may also well account for the recent report of a surprisingly high frequency of CD4\(^{\alpha\alpha}\) T cells that respond specifically to pMHC but are not stained by the corresponding tetramers (13). The frequency of such tetramer-negative, functionally competent T cells would be expected to be higher in CD4\(^{\alpha\alpha}\) than in CD8\(^{\alpha\alpha}\) T cell populations because of differences in their coreceptor ectodomain binding to MHC; CD8 generally binds weakly to classical MHC but CD4 binds hardly at all to class II MHC (46, 47).

It is likely that the oligomers bind stably (multivalently) only to those TCR molecules that are closely clustered. The spacing between biotin (pMHC) binding sites on SA is 2–4 nm (48), whereas TCR, if uniformly distributed on the cell surface, would generally be much further apart (separated on average by ~60–70 nm on a T cell of 8.5 μm diameter with 56,000 TCR molecules per cell [Fig. 2E, Table I]). But cell-surface TCR, like many other integral membrane proteins, are aggregated into groups (“islands”), some depending upon cholesterol (lipid rafts) for their clustering (15, 49–51; reviewed in Ref. 52). Whether the SA-oligomers bind selectively to particular TCR clusters is not clear. The stronger binding of pMHC dimers to activated than to naive T cells and abolition of this difference by reducing cholesterol content of the cell membrane (15) indicate that oligomer binding has the potential to be developed into a useful procedure to define the size and character of TCR clusters on cells that differ functionally or in developmental status.

For the TCR subset that can stably engage SA-oligomers, an apparent equilibrium constant for the oligomer–TCR interaction \((K_{D,\text{olig}})\) can be defined as the free SA-oligomer concentration that...
CD8 effect can be increased up to 8-fold because most for SA-linked oligomers, which are effectively trimeric, the lifetime of the TCR–pMHC bond only modestly, 2-fold at the CD8 ectodomain’s binding to the MHC (32) and computational analysis (46) concur in showing that in the trimolecular CD8–pMHC–TCR reaction. Experimental findings under three-dimensional conditions (2, 3). Exceptionally fast and their apparent affinities much higher than for studies have reported the kinetics of such 2D interactions to be quasi 2D interactions that occur under physiological conditions of Scatchard plots (1, 15) and by the Sips distribution (53). Stably (multivalently) constitute a small fraction of all cell surface of the presumably clustered TCR molecules that bind SA-oligomers and CD8 pos T cells and the much higher values found with Ig-based dimers and SA-oligomers on CD8 pos T cells. Although the presumably clustered TCR molecules that bind SA-oligomers stably (multivalently) constitute a small fraction of all cell surface TCR (41), they and unclustered TCR have the same intrinsic (monovalent) affinity. This uniformity is indicated by the linearity of Scatchard plots (1, 15) and by the Sips distribution (53).

The binding of pMHC oligomers to cell-surface TCR approximates more closely than the other conditions in Table IV to the quasi 2D interactions that occur under physiological conditions at the interface of T cells and target cells or other APCs. Recent studies have reported the kinetics of such 2D interactions to be exceptionally fast and their apparent affinities much higher than for the same interactions under three-dimensional conditions (2, 3).

Finally, it is worth commenting on the contribution of CD8αβ in the trimolecular CD8–pMHC–TCR reaction. Experimental findings (32) and computational analysis (46) concur in showing that the CD8 ectodomain’s binding to the MHC α3 domain increases the lifetime of the TCR–pMHC bond only modestly, 2-fold at most. For SA-linked oligomers, which are effectively trimers, the CD8 effect can be increased up to 8-fold because kff is proportional to koff (Eq. 2 and Refs. 32, 46). The CD8 effect on oligomers that expressed the high-affinity TCR (m33) is consistent with these values: t1/2 is ~1.4-fold greater for the CD8 pos than CD8 neg cells (Fig. 3A). However, for oligomers that express the lower affinity 2C TCR, t1/2 is 20-fold greater for the CD8 pos than the CD8 neg cells, which exceeds the estimated upper limit of eight and reflects the greater dissociative losses of bound oligomers from relatively low-affinity TCR (Fig. 3C). The still far greater difference, ~50-fold, between the CD8 pos hybridoma and CD8 pos splenic T cells that express the same TCR (2C) (t1/2 ~0.1 min versus 5.85 min), likely arises from the additional effect of the ~20-fold greater surface density of the TCR on the splenic T cells, resulting in an increase in the multivalent on-rate (μ) and from the proportionality of koff to μ (Eq. 2).

### Appendix A: Model Equations

#### Dissociation of bound oligomers

\[ L \rightleftharpoons \frac{3k_{off}}{\mu} \] 
\[ \frac{d[L - T]}{dt} = -k_{off}[L - T] + 2k_{off}[L = T] - 2\mu[L - T], \quad \text{(A1)} \]

\[ \frac{d[L = T]}{dt} = -2k_{off}[L = T] - \mu[L = T] + 2\mu[L - T] \]
\[ + 3k_{off}[L = T], \quad \text{(A2)} \]

and

\[ \frac{d[L = T]}{dt} = -3k_{off}[L = T] + \mu[L = T]. \quad \text{(A3)} \]

#### Derivation of text Eq. 2 and 5

**Text Eq. 2.** Concentration of oligomers (L) bound to cell-surface TCR (T) is equal to the sum of all bound forms:

\[ L_B = L - T + L = T + L = T. \quad \text{(A4)} \]
Loss of bound oligomers is determined by dissociation of the singly bound form:

\[
\frac{dL_B}{dt} = -k_{off}L_B = -k_{off}[L - T].
\]  

(A5)

As our oligomer dissociation data exhibit exponential decay kinetics, we can safely assume a rapid equilibrium and interconvertibility (mass equilibrium) of bound forms, as has been assumed previously (32). Applying this assumption, we get:

\[
[L = T] = \frac{\mu}{k_{off}}[L - T], \text{ and}
\]

(A6)

\[
[L = T] = \frac{\mu}{3k_{off}}[L - T] = \frac{\mu^2}{3k_{off}^2}[L - T].
\]  

(A7)

Plugging in (A6-7) to (A4):

\[
L_B = [L - T] \left(1 + \frac{\mu}{k_{off}} + \frac{\mu^2}{3k_{off}^2}\right) = [L - T] \left(3k_{off} + 3k_{off}\mu + \mu^2\right).
\]

(A8)

Plugging (A8) into (A5) yields Text Eq. 2:

\[
\frac{L_{bound}}{L_{bound3}} = \exp[-k_{off}t] = \exp\left[-\frac{3k_{off} + 3k_{off}\mu + \mu^2}{k_{off}^2}t\right] \approx \exp\left[-\frac{k_{off}^3}{k_{off}^2}t\right],
\]

where the approximate equality can be applied under the conditions where \(\mu >> k_{off}\), as is true for all of the interactions described in this study. Where \(\mu \leq k_{off}\), the roots of the quadratic equation for \(\mu\) can be solved.

**Derivation of Text Eq. 5**

At equilibrium, the rate of oligomer association is equal to the rate of oligomer dissociation:

\[
k_{on}T_{free}L_{sol} = k_{off}L_{bound}.
\]

where \(k_{on}\) is on-rate (\(k_{on}\)) for the monomeric pMHC-TCR interaction, as measured by instance by SPR, modified by the number of pMHC per streptavidin molecule.

If 0 is taken to be the fraction of occupied receptors, \(T_{free} = (1 - 0)T_{total}\) and \(L_{bound} = 0\) \(T_{total}\). Eq. 5 from the main text then follows:

\[
\frac{k_{on}T_{free}L_{sol}}{k_{off}L_{bound}} = \frac{k_{on}T_{free}L_{sol}^{k_{off}}} {L_{bound}^{k_{off}}},
\]

where \(k_{on}\) is on-rate (\(k_{on}\)) for the monomeric pMHC-TCR interaction, as measured by instance by SPR, modified by the number of pMHC per streptavidin molecule.

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\]

where \(k_{on}\) is on-rate (\(k_{on}\)) for the monomeric pMHC-TCR interaction, as measured by instance by SPR, modified by the number of pMHC per streptavidin molecule.

If 0 is taken to be the fraction of occupied receptors, \(T_{free} = (1 - 0)T_{total}\) and \(L_{bound} = 0\) \(T_{total}\). Eq. 5 from the main text then follows:

\[
\frac{k_{on}T_{free}L_{sol}}{k_{off}L_{bound}} = \frac{k_{on}T_{free}L_{sol}^{k_{off}}} {L_{bound}^{k_{off}}},
\]

where \(k_{on}\) is on-rate (\(k_{on}\)) for the monomeric pMHC-TCR interaction, as measured by instance by SPR, modified by the number of pMHC per streptavidin molecule. If 0 is taken to be the fraction of occupied receptors, \(T_{free} = (1 - 0)T_{total}\) and \(L_{bound} = 0\) \(T_{total}\). Eq. 5 from the main text then follows:

\[
\frac{k_{on}T_{free}L_{sol}}{k_{off}L_{bound}} = \frac{k_{on}T_{free}L_{sol}^{k_{off}}} {L_{bound}^{k_{off}}},
\]

where \(k_{on}\) is on-rate (\(k_{on}\)) for the monomeric pMHC-TCR interaction, as measured by instance by SPR, modified by the number of pMHC per streptavidin molecule.

If 0 is taken to be the fraction of occupied receptors, \(T_{free} = (1 - 0)T_{total}\) and \(L_{bound} = 0\) \(T_{total}\). Eq. 5 from the main text then follows:

\[
\frac{k_{on}T_{free}L_{sol}}{k_{off}L_{bound}} = \frac{k_{on}T_{free}L_{sol}^{k_{off}}} {L_{bound}^{k_{off}}},
\]

where \(k_{on}\) is on-rate (\(k_{on}\)) for the monomeric pMHC-TCR interaction, as measured by instance by SPR, modified by the number of pMHC per streptavidin molecule.

If 0 is taken to be the fraction of occupied receptors, \(T_{free} = (1 - 0)T_{total}\) and \(L_{bound} = 0\) \(T_{total}\). Eq. 5 from the main text then follows:

\[
\frac{k_{on}T_{free}L_{sol}}{k_{off}L_{bound}} = \frac{k_{on}T_{free}L_{sol}^{k_{off}}} {L_{bound}^{k_{off}}},
\]

where \(k_{on}\) is on-rate (\(k_{on}\)) for the monomeric pMHC-TCR interaction, as measured by instance by SPR, modified by the number of pMHC per streptavidin molecule.

If 0 is taken to be the fraction of occupied receptors, \(T_{free} = (1 - 0)T_{total}\) and \(L_{bound} = 0\) \(T_{total}\). Eq. 5 from the main text then follows:

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\frac{k_{on}T_{free}L_{sol}}{k_{off}L_{bound}} = \frac{k_{on}T_{free}L_{sol}^{k_{off}}} {L_{bound}^{k_{off}}},
\]

where \(k_{on}\) is on-rate (\(k_{on}\)) for the monomeric pMHC-TCR interaction, as measured by instance by SPR, modified by the number of pMHC per streptavidin molecule.

If 0 is taken to be the fraction of occupied receptors, \(T_{free} = (1 - 0)T_{total}\) and \(L_{bound} = 0\) \(T_{total}\). Eq. 5 from the main text then follows:

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\]

where \(k_{on}\) is on-rate (\(k_{on}\)) for the monomeric pMHC-TCR interaction, as measured by instance by SPR, modified by the number of pMHC per streptavidin molecule.

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\]

where \(k_{on}\) is on-rate (\(k_{on}\)) for the monomeric pMHC-TCR interaction, as measured by instance by SPR, modified by the number of pMHC per streptavidin molecule.

If 0 is taken to be the fraction of occupied receptors, \(T_{free} = (1 - 0)T_{total}\) and \(L_{bound} = 0\) \(T_{total}\). Eq. 5 from the main text then follows:

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\]

where \(k_{on}\) is on-rate (\(k_{on}\)) for the monomeric pMHC-TCR interaction, as measured by instance by SPR, modified by the number of pMHC per streptavidin molecule.