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

Jennifer D. Stone,∗,1 Maxim N. Artyomov,†,‡,§,¶,† Adam S. Chervin,*
Arup K. Chakraborty,†,‡,§,¶,‖ Herman N. Eisen,‖,¶ and David M. Kranz*†

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

Abbreviations used in this article: 2D, two-dimensional; pMHC, peptide–MHC; scTCR, single chain Vβ-linker-Vα TCR; SPR, surface plasmon resonance.

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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 ($\mu$) 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-KK 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 280 nm and 290 nm (PBS [pH 7.4]), using a molar extinction coefficient of 210,000 M$^{-1}$ cm$^{-1}$ for fluorescein. The rapidity of dissociation of tetrafluorescein-labeled SA was added stepwise to the biotinylated SIY/Kb molecule was determined by comparing the ratio of UV-Vis absorbance at 280 nm to yield protein absorbance and concentration. Multiple independent dilutions of each protein were scanned to determine the fluorescein to protein ratio. Protein molar extinction coefficients were absorbance at 280 nm to yield protein absorbance and concentration.

Results

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/KK 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.

Model

To describe the behavior of SA-linked oligomers binding to and dissociating from T cells, we developed and applied a quantitative model. 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 $L$, $L_T$, $L_T$, and $L_T$. Where $L$ refers to oligomer (ligand) and $T$ to TCR. As described below (in Results) and shown in detail in Appendix A: Model Equations, a bound oligomer’s intrinsic dissociation rate is determined by $k_D$, and the intrinsic association rate of the pMHC–TCR bond, as measured, for example, by SPR and $\mu$ (a multivalent $k_w$ with units of s$^{-1}$), which is related to the intrinsic [univalent] pMHC–TCR association rate, $k_w$ [with units of M$^{-1}$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: $k_D L_T = k_D L_T$, where $k_D$ 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, $T_{free} = (1 - \theta) L_{total}$ and $L_{bound} = \theta L_{total}$, 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 $\mu > k_{off}$ as is the case
for the TCR–pMHC interactions described in this study ($\mu \sim 10-40$-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 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/Kβ: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 successively 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/Kβ 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+ 2C T cells freshly isolated from spleens of 2C TCR transgenic mice (Fig. 1C). CD8α and CD8β 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 ($K_{off}$) 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/Kβ oligomers bound equally well to 1) hybridoma cells (58%) 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 CD8αβ to peptide-Kβ [$K_\beta = 38 \mu M$ (35)] and 2C TCR to SIY-Kβ [$K_\beta = 30 \mu M$ (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 ($K_{D, olig}$). The ratio between $K_{D, olig}$ 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 CD8α and CD8β hybridomas (Fig. 1B). However, for the much lower affinity wild-

**FIGURE 1.** MHC oligomer staining of retrovirally-transduced T cell hybridomas. A, Distribution of oligomeric species in oligomer (tetramer) preparations. Reduced, unboiled gradient SDS-PAGE gel of reaction of larger than $k_{off}$ site specifically on the H chain concurrently with translation by biotin ligase-expressing E. coli, with a modification efficiency of ~50%. The following experiments were carried out using reagent corresponding to 16:1 MHC:SA ratio. B, Steady-state oligomer staining of T cell hybridomas with and without CD8αβ expression—corrected by subtracting fluorescence of 58% cells without TCR or CD8 at the same conditions. C, Steady-state staining of 2C transgenic T cells carried out at 4°C using various concentrations of SIY/Kβ:SA-oligomer. Binding of SIY/Kβ:SA-oligomer to C57BL/6 T cells was subtracted from binding to 2C transgenic T cells. Quantification of molecules per cell indicates number of fluorescent SAs associated with the cell; actual number of MHC-bound TCRs may be up to three times higher.

The dissociation data (32). In a range where this assumption is not valid, including much more weakly binding receptors, 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 $\mu > k_{off}$, $k_{off}$ 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.
type 2C TCR, the half-maximal concentrations with CD8
neg 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
Abs to various TCR domains (C
β and V
β8) 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 summa-
rized 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 mono-
mer (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/K
b oligomers from oligomer-stained cells
(Fig. 3A, 3B). The steps involved in dissociation can be repre-
sented by:

![Figure 2](http://www.jimmunol.org/)
Table I. Cell surface density of TCR and CD8 on splenic T cells and transduced T cell hybridomas

<table>
<thead>
<tr>
<th>T Cells</th>
<th>TCR CJb(^a)</th>
<th>TCR Vβ8(^b)</th>
<th>CD3e(^c)</th>
<th>2C TCR (Clonotype)(^d)</th>
<th>CD8α(^f)</th>
<th>CD8β(^f)</th>
<th>Average No. TCR per cell ((x \times 10^5))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8αβ only</td>
<td>0.2</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>57.4</td>
<td>78.9</td>
<td>0</td>
</tr>
<tr>
<td>2C TCR</td>
<td>6.8</td>
<td>8.7</td>
<td>9.8</td>
<td>7.5</td>
<td>−0.1</td>
<td>0.2</td>
<td>15.3</td>
</tr>
<tr>
<td>2C TCR + CD8αβ</td>
<td>9.7</td>
<td>13.5</td>
<td>15.4</td>
<td>6.3</td>
<td>85.8</td>
<td>117</td>
<td>19.7</td>
</tr>
<tr>
<td>m33 TCR</td>
<td>9.3</td>
<td>12.4</td>
<td>13.7</td>
<td>−0.8</td>
<td>−0.01</td>
<td>−0.03</td>
<td>21.7</td>
</tr>
<tr>
<td>m33 TCR + CD8αβ</td>
<td>11.0</td>
<td>14.5</td>
<td>16.7</td>
<td>−0.4</td>
<td>0.14</td>
<td>140</td>
<td>25.5</td>
</tr>
<tr>
<td>CD8(^e) splenic T cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>25.6</td>
<td>33.0</td>
<td>41.0</td>
<td>0.4</td>
<td>69.3</td>
<td>84.0</td>
<td>58.2</td>
</tr>
<tr>
<td>2C TCR transgenic</td>
<td>26.7</td>
<td>36.5</td>
<td>42.2</td>
<td>21.4</td>
<td>46.7</td>
<td>58.3</td>
<td>56.4</td>
</tr>
</tbody>
</table>

\(^{a}\)557-597 Ab (BD Pharmingen).
\(^{b}\)F23.1 Ab (BD Pharmingen).
\(^{c}\)145-2C11 Ab (BD Pharmingen).
\(^{d}\)B2 Ab labeled with FITC.
\(^{e}\)53-6.7 Ab (BD Pharmingen).
\(^{f}\)Estimated maximum number of TCR per cell line taken twice as the average of TCR CJb, TCR Vβ8, and 2C TCR (for 2C TCR transgenic or transduced T cells).

\[ L = T \frac{3k_{\text{off}}}{{\mu}} - L = T \frac{2k_{\text{off}}}{{2\mu}} - L \to k_{\text{off}} \to L_{\text{sol}}. \]  
(1)

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 oligomers 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 constraints) 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 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

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

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

As seen in Fig. 3A, 3B, the dissociation rates from each of the hybridomas and T cells could indeed be fitted by a single-value exponential decay constant \( (k_{\text{off}}) \), which is equivalent to \( k_{\text{off}}^{3} \) \( (3k_{\text{off}}^{2} + 3k_{\text{off}} + \mu^{2}) \) 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 \( k_{\text{off}} \) values (Fig. 3), the amounts of oligomer lost during the ~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\(^{pos}\) splenic T cells. These corrections were based initially on dissociation rates measured at 25°C (Fig. 3A, 3B). But, as the cells were washed in the cold (~10°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\(^c\) 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 \( k_{\text{off}} \) measured at 25°C, to estimate \( k_{\text{off}} \) 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., \( \mu = k_{\text{on}} \times \) 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

\[ \mu_{10'C} = \mu_{25'C} \frac{k_{\text{on},10'C}}{k_{\text{on},25'C}}. \]  
(3)

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, \( k_{\text{off}} \) 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 ~10°C indicate that before oligomer-stained cells were washed only ~15% of the TCR molecules on splenic CD8\(^{pos}\) T cells and ~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 \( (k_{\text{off,app}}) \) and the time spent preparing cells for flow cytometry (Fig. 3C). Because \( k_{\text{off}} \) 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 CD8aβg hybridoma, dissociation was so fast that the rate could hardly be measured. However, with CD8αg hybridoma that expressed the same TCR at about the same level, t1/2,app increased to >2 min (Fig. 3A and inset). For hybridomas that expressed the high-affinity m33 TCR, the oligomer dissociation t1/2 increased from 5.9 min on CD8αg cells to 8.5 min on CD8αg cells (Fig. 3B).

To compare T cell hybridomas with T cells freshly isolated from mice, CD8αg T cells from Rag−/− 2C TCR transgenic splenocytes were examined side-by-side with CD8αg m33 TCR+ 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 CD8αg 2C T cells than from CD8αg hybridomas that express the same TCR (t1/2,app = 5.85 and 2.1 min, respectively) (Fig. 3A, 3B)? The answer likely lies in the much greater number of TCR per cell 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 koff 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_D,olig)**

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:

\[
L_{sol} + T \rightleftharpoons k_{on} L + T \rightleftharpoons \frac{2μ}{2k_{off}} L \rightleftharpoons \frac{μ}{3k_{off}} L = T, \tag{4}
\]

where all of the terms are defined as in Eq. 1 except for k'_{on}, which refers to the on-rate for the first pMHC of the oligomer to bind. We take k'_{on} 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,

\[
\frac{θ}{1 θ} = \frac{k'_{on}L_{sol}(3k_{off}^2 + 3k_{off}μ + μ^2)}{k_{off}^3} = \frac{k'_{on}L_{sol}}{k_{off}^3}. \tag{5}
\]

where L_{sol} 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.
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From the titration shown in Fig. 1C, the apparent affinity ($K_{D,\text{olig}}$) of the 2C TCR on splenic CD8$^\text{pos}$ T cells for SA-SIY/K$^b$ oligomers is $\sim$1 nM, as determined by the free oligomer concentration at 50% maximum bound. Comparisons of $K_{D,\text{olig}}$ to monovalent K$^d$ are useful to demonstrate the benefits of multivalent ligand binding to TCR (e.g., the $K_{D}/K_{D,\text{olig}}$ ratio has been termed an enhancement factor) (19, 45).

**Discussion**

This study confirms previous indications that SA-based pMHC oligomers (tetramers) bind stably to only a fraction of cognate cell surface TCR (19). The fraction amounted to $\sim$15% of the TCR on CD8$^\text{pos}$ splenic T cells from 2C TCR transgenic mice and $\sim$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 CD8$^\text{pos}$ 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$^\text{pos}$ 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$^\text{pos}$ than in CD8$^\text{pos}$ T cell populations because of differences in their coreceptor ectodomain binding to MHC; CD8 generally binds weakly to class I 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 $\sim$60–70 nm on a T cell of 8.5 $\mu$m 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 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 membranes (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

**Table II. Extent of TCR occupancy by SA-linked MHC oligomers**

<table>
<thead>
<tr>
<th>T Cells</th>
<th>Maximum No. TCR Engaged by pMHC Oligomers$^a$</th>
<th>Measured 25°C</th>
<th>10°C$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total No. TCR per cell$^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2C TCR</td>
<td>15,300</td>
<td>2985</td>
<td>0.20</td>
</tr>
<tr>
<td>2C TCR and CD8$\alpha$$\beta$</td>
<td>19,700</td>
<td>2058</td>
<td>0.10$^c$</td>
</tr>
<tr>
<td>m33 TCR</td>
<td>21,700</td>
<td>6225</td>
<td>0.29$^c$</td>
</tr>
<tr>
<td>m33 TCR and CD8$\alpha$$\beta$</td>
<td>25,500</td>
<td>6309</td>
<td>0.25$^c$</td>
</tr>
<tr>
<td>CD8$^+$ splenic T cells</td>
<td>58,200</td>
<td>(450)</td>
<td>—</td>
</tr>
<tr>
<td>2C TCR transgenic</td>
<td>56,400</td>
<td>7050</td>
<td>0.13$^c$</td>
</tr>
</tbody>
</table>

The extent of these losses during an 8 min wash carried out at 25°C (Table III).

**Table III. Binding of SIY/K$^b$ monomer by various TCR measured by SPR at different temperatures**

<table>
<thead>
<tr>
<th>TCR</th>
<th>$k_a$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_{\text{off}}$ (s$^{-1}$)</th>
<th>$K_D$ (nM)</th>
<th>$k_{\text{eff,olig}}$ (min$^{-1}$)$^a$</th>
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<tbody>
<tr>
<td>m33</td>
<td>2.5 × 10$^5$</td>
<td>0.0036</td>
<td>17.1</td>
<td>0.008</td>
</tr>
<tr>
<td>S51</td>
<td>3.5 × 10$^5$</td>
<td>0.0013</td>
<td>3.97</td>
<td>0.001</td>
</tr>
<tr>
<td>Y26</td>
<td>3.3 × 10$^5$</td>
<td>0.0023</td>
<td>7.2</td>
<td>0.004</td>
</tr>
<tr>
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<td>8.6</td>
<td>0.001</td>
</tr>
<tr>
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<td>2.8 × 10$^5$</td>
<td>0.0036</td>
<td>12.7</td>
<td>0.004</td>
</tr>
<tr>
<td>S51/Y48</td>
<td>1.7 × 10$^5$</td>
<td>0.051</td>
<td>305</td>
<td>0.29</td>
</tr>
<tr>
<td>Y48</td>
<td>1.65 × 10$^5$</td>
<td>0.185</td>
<td>1130</td>
<td>0.12</td>
</tr>
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$^a$Measurements of monovalent binding properties carried out at 10°C by SPR.

$^b$Values of binding of SIY/K$^b$ at 25°C reprinted with permission from Chervin et al. (19).

$^c$k$_{\text{off,olig}}$ at 10°C estimated from monovalent binding properties and from $k_{\text{eff,olig}}$ values at 25°C, using Eq. (2) and the relationship: $\mu_{25^\circ C} = \mu_{21^\circ C} \times k_{\text{off,olig,25^\circ C}}/k_{\text{off,olig,21^\circ C}}$.
CD8 effect can be increased up to 8-fold because the lifetime of the TCR–pMHC bond only modestly, 2-fold at a
in the trimolecular CD8–pMHC–TCR reaction. Experimental find-
the same interactions under three-dimensional conditions (2, 3).
ceptionally fast and their apparent affinities much higher than for
studies have reported the kinetics of such 2D interactions to be ex-
quasi 2D interactions that occur under physiological conditions
of Scatchard plots (1, 15) and by the Sips distribution (53).
( monovalent) affinity. This uniformity is indicated by the linearity
the presumably clustered TCR molecules that bind SA-oligomers
and CD8 pos splenic T cells that express the same TCR (2C) (Fig.
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 \( k_{off}^{eff} \) to \( k_{off}^{3} \mu^{-1} \) (Eq. 2).

**Appendix A: Model Equations**

**Dissociation of bound oligomers**

\[
L = T \frac{3k_{off}^{eff}}{\mu} L = T \frac{2k_{off}}{2\mu} L - T \frac{k_{off}}{L_{sol}}, \quad \text{(Scheme 1)}
\]

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 \( (L_{sol}) \).

We assume that for each pMHC–TCR interaction, \( k_{off} \) and \( \mu \) (the
multivalent on rate, see text) are independent of the binding
of neighboring MHC with TCR. Thus, stoichiometric coefficients in
front of \( k_{off} \) and \( \mu \) correspond to the number of pMHCs available
for binding/unbinding.

The following differential equations were used to describe intercon-
version among bound forms and loss of bound forms in accord with Scheme (1):

\[
d[L - T] = -k_{off}^{eff}[L - T] + 2k_{off}^{eff}[L = T] - 2\mu[L - T], \quad \text{(A1)}
\]

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

and

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

**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:

\[ \frac{dL_B}{dt} = -k_{off}\frac{L}{k_{on}L_{sol}} \]  

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

Applying this assumption, we get:

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

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

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

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

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

\[ \text{Plugging (A8) in to (A5) yields Text Eq. 2: }\]

\[ \frac{L_{bound}}{L_{bound0}} = \exp\left[-k_{off}t\right] \]

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

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:

\[ k_{on}T_{free}L_{sol} = k_{off}L_{bound0} \]

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

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

\[ 0 = k'_{on}L_{sol}(3k_{off} + k_{off} \mu + \mu^2) = k_{off}L_{bound0} \]

Eq. 5 resembles the Scatchard equation. That equation, arguably the most widely used one in immunology, was originally developed (30) to account for the equilibrium binding of small molecules and ions to proteins. For ligand–protein interactions, it is usually expressed as \( r/(n-r) = K_r \), where \( r \) represents moles bound ligand, \( n \) the moles of ligand maximally bound, \( c \) the free ligand concentration, and \( K \) the equilibrium (association) constant.

Independent derivations of Scatchard’s equation have generally been based upon the distribution of bound and free ligands at equilibrium (29, 31). In contrast, Eq. 5 above was derived from the kinetics of multivalent ligand binding to cell surface receptors. If, however, Eq. 5 were applied to monovalent ligand–receptor interactions, \( \mu \) would become zero, \( k_{off} \) would correspond to the intrinsic association rate, \( k_{on} \), and Eq. 5 would then be equivalent to the Scatchard equation.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


