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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 associative 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.

Becaus 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 saturation 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 (SIYRYYGL) 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-Kb 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 supernatant using protein G beads. 1B2 was labeled with FITC, and purified to remove excess FITC. For each fluorescein-labeled protein, the concentration of fluorescein-labeled SA-SIY/K b oligomers in FACS tubes from some TCR can account for the frequency of tetramer-negative, functionally competent T cells.

**Protein expression and preparation**

Single chain V8-linker-Va 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 b chain containing a C-terminal biotinylation signal peptide and human b2 microglobulin L chains were expressed separately in E. coli.**

H2-K b 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 (19). Folded complexes were purified by anion exchange chromatography using HiTrap Q columns (GE Healthcare, Piscataway, NJ) and size exclusion chromatography. For incorporation of SIY/K b into SA-oligomers, precharacterized, calibrated, fluorescein-labeled SA was added stepwise to the biotinylated SIY/K b 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. The 2C transgenic and C57BL/6 mouse splenocytes were incubated with various concentrations of fluorescein-labeled SA-SIY/K b oligomers 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−/− cell line (control) were subtracted as background from the TCR transfected hybridoma values at the same staining concentration.

**Oligomer dissociation experiments**

To perform steady-state oligomer binding titrations, 58−/− 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/K b oligomers 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−/− cell line (control) were subtracted as background from the TCR transfected hybridoma values at the same staining concentration.

**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/K b 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 described below (in Results) and shown in detail in Appendix A: Model Equations, a bound oligomer’s dissociation rate is dependent on the association rate for fluid phase (FCS) $k_{1s}$ and for dissociation rate constant for cell-bound $k_{off}$ (the intrinsic dissociation rate of the pmh–TCR–bond, as measured, for example, by SPR) and $\mu$ (a multivalent $k_{b}$ with units of $s^{-1}$), which is related to the intrinsic [univalent] pmh–TCR association rate, $k_{on}$ [with units of $M^{-1} \cdot s^{-1}$], 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 $k_{on} \cdot [T] \cdot [O] = \frac{k_{off}}{[O]_{bound}}$, where $k_{on}$ 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, $T_{free} = (1 − \theta)[T]_{total}$ 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 deriving 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 (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 pronounced for low-affinity than high-affinity TCR–pMHC interactions (19). It was thus not surprising that for m33, the very high-affinity interactions (19, 21, 23, 24) for low-affinity wild-type K^b 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-K^b [KD = 38 μM (35)] and 2C TCR to SIY-K^b [KD = 30 μM (19, 23, 24)].

The TCR affinity for the oligomer is generally taken to be the free oligomer concentration when the amount of bound oligomers is half of the plateau or maximal level (KD_{olig}). The ratio between KD_{olig} and the equilibrium constant (K_{D}) for monovalent binding of the same TCR to the same pMHC as monomer (KD_{monomer}/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 pos and CD8 neg 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^- 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 (KD_{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%) 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-K^b [KD = 38 μM (35)] and 2C TCR to SIY-K^b [KD = 30 μM (19, 23, 24)].
type 2C TCR, the half-maximal concentrations with CD8\textsuperscript{neg} and CD8\textsuperscript{pos} 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 (C\textsubscript{B} and V\textsubscript{B}) 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/K\textsuperscript{b} oligomers from oligomer-stained cells (Fig. 3A, 3B). The steps involved in dissociation can be represented by:

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**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 C\textsubscript{B} (clone H57-597), TCR V\textsubscript{B} (clone F23.1), CD3\textsubscript{e} (clone 145-2C11), the 2C TCR specifically (clone 1B2), and (B, D) CD8\textsubscript{a} (clone 53-6.7) and CD8\textsubscript{b} (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).
From the experimentally determined $k_{off}^{eff}$ 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<sup>pos</sup> 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/KB 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 $k_{off}^{eff}$ measured at 25°C, to estimate $k_{off}^{eff}$ at 10°C.

As is discussed in Appendix A: Model Equations, the difference in dimensions of $\mu$ (measured in s<sup>-1</sup>) and intrinsic monovalent on-rates, $k_{on}$ (which are measured for reactants in solution and expressed in M<sup>-1</sup>s<sup>-1</sup>) 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., $\mu = k_{on} \times$ local surface density of TCR, where $k_{on}$ is essentially the monovalent $k_{on}$ 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

$$\mu_{10°C} = \frac{k_{on,10°C}}{k_{on,25°C}} \mu_{25°C}. \quad (3)$$

Values for $k_{on}$ at the two temperatures are shown in Table III for m33 and several other engineered mutants of the 2C TCR. From these values, $k_{off}^{eff}$ 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 ~10°C indicate that before oligomer-stained cells were washed only ~15% of the TCR molecules on splenic CD8<sup>pos</sup> 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 ($k_{off,app}$) and the time spent preparing cells for flow cytometry (Fig. 3C). Because $k_{off}$ and intrinsic TCR–pMHC affinity ($K_{d}$) 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 CD8αβ hybridoma, dissociation was so fast that the rate could hardly be measured. However, with CD8αβ hybridoma that expressed the same TCR at about the same level, $t_{1/2}\text{app}$ increased to >2 min (Fig. 3A and inset). For hybridomas that expressed the high-affinity m33 TCR, the oligomer dissociation $t_{1/2}$ increased from 5.9 min on CD8αβ cells to 8.5 min on CD8αβ cells (Fig. 3B). To compare T cell hybridomas with T cells freshly isolated from mice, CD8αβ T cells from Rag–/– 2C TCR transgenic splenocytes were examined side-by-side with CD8αβ 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 CD8αβ 2C T cells than from CD8αβ hybridomas that express the same TCR ($t_{1/2}\text{app} = 5.85$ and 2.1 min, respectively) (Fig. 3A, 3B)? The answer likely lies in the much greater number of TCR per cell on the transduced hybridomas, the greater stability of the SA-oligomer equilibrium binding constants ($k_{\text{off}}$), and smaller $k_{\text{eff}}$ (larger $t_{1/2}\text{app}$) 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 $k_{\text{off}}$ 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 ($\mu$) on the T cells.

**Oligomer equilibrium binding constants ($K_{D,\text{olig}}$)**

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:

$$L_{\text{sol}} + T \xrightleftharpoons[k_{\text{off}}]{k_{\text{on}}} L \xrightleftharpoons[k_{\text{off}}]{k_{\text{on}}} L = T \xrightleftharpoons[k_{\text{off}}]{\mu} L = T,$$

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

$$\frac{\theta}{1 - \theta} = \frac{k_{\text{on}} L_{\text{sol}}(3k_{\text{off}}^2 + 3k_{\text{off}}\mu + \mu^2)}{k_{\text{off}}^3} = k_{\text{on}} L_{\text{sol}} k_{\text{off}}^{-3},$$

where $L_{\text{sol}}$ is the concentration of oligomers in solution, and $\theta$ 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.
which TCR binds oligomers weakly (large

to TCR–pMHC affinity (Fig. 3 and Eq. 2). For T cells for

C

for reports of tetramer-negative functionally competent CD8 pos

to commonplace washing conditions. This effect could account

K

valent ligand binding to TCR (e.g., the

K

from Table I.

K

Maximum level of MHC oligomer saturation measured, allowing for three MHC engaged per SA-oligomer.

K

Maximum TCR occupancy estimated at steady-state correcting for losses during a preanalysis wash carried out at 25°C.

K

Maximum TCR occupancy estimated at steady-state correcting for losses during a preanalysis wash carried out at 10°C.

Conversion of koff,olig for 2C uses koff and koff for 2C measured at 25°C, but koff and koff for the closely-related TCR Y48 measured at 10°C (Table III).

Discussion

This study confirms previous indications that SA-based pMHC oligomers (tetramers) bind stably to only a fraction of cognate cell surfaces (19). The fraction amounted to ∼15% of the TCR on CD8pos splenic T cells from 2C TCR transgenic mice and ∼10% of the 2C TCR on splenic CD8pos T cells (19, 45). From Table III.

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

<table>
<thead>
<tr>
<th>T Cells</th>
<th>Maximum No. TCR Engaged/Total No. TCR</th>
<th>Corrected for Losses during 8 Min Wash at:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>25°C</td>
</tr>
<tr>
<td>T cell hybridomas expressing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2C TCR</td>
<td>15,300</td>
<td>2985</td>
</tr>
<tr>
<td>2C TCR and CD8αβ</td>
<td>19,700</td>
<td>2058</td>
</tr>
<tr>
<td>m33 TCR</td>
<td>21,700</td>
<td>6225</td>
</tr>
<tr>
<td>m33 TCR and CD8αβ</td>
<td>25,500</td>
<td>6309</td>
</tr>
<tr>
<td>CD8+ splenic T cells</td>
<td>58,200</td>
<td>(450)</td>
</tr>
<tr>
<td>2C TCR transgenic</td>
<td>56,400</td>
<td>7050</td>
</tr>
</tbody>
</table>

From the titration shown in Fig. 1C, the apparent affinity (K_D,olig) of the 2C TCR on splenic CD8pos T cells for SA-SIY/Kb oligomers is ∼1 nM, as determined by the free oligomer concentration at 50% maximum bound. Comparisons of K_D,olig to monovalent K_f are useful to demonstrate the benefits of multivalent ligand binding to TCR (e.g., the K_D/K_D,olig ratio has been termed an enhancement factor) (19, 45).

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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 ∼15% of the TCR on CD8pos splenic T cells from 2C TCR transgenic mice and ∼40% on a TCR-transduced hybridoma line. These less-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_f,olig, which is related to TCR–pMHC affinity (Fig. 3C and Eq. 2). For T cells for which TCR binds oligomers weakly (large K_f,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 functionality competent CD8pos T cells (9–12). Losses of bound oligomers by dissociation from low-affinity TCR may also well account for the recent report of SIY/Kb monomer by various TCR measured by SPR at different temperatures

<table>
<thead>
<tr>
<th>TCR</th>
<th>k on (M−1s−1)</th>
<th>k off (s−1)</th>
<th>K D (nM)</th>
<th>k off,olig (min−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>m33</td>
<td>2.5 × 10^5</td>
<td>0.0036</td>
<td>17.1</td>
<td>0.008</td>
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<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>
<td>Y49</td>
<td>2.3 × 10^5</td>
<td>0.0019</td>
<td>8.6</td>
<td>0.001</td>
</tr>
<tr>
<td>N27</td>
<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>
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</table>

<table>
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<tr>
<th>TCR</th>
<th>k on (M−1s−1)</th>
<th>k off (s−1)</th>
<th>K D (nM)</th>
<th>k off,olig (min−1)</th>
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<tr>
<td>m33</td>
<td>13.7 × 10^5</td>
<td>0.015</td>
<td>16</td>
<td>0.08</td>
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<td>5.15 × 10^5</td>
<td>0.008</td>
<td>15</td>
<td>0.05</td>
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<td>8.5 × 10^5</td>
<td>0.013</td>
<td>17</td>
<td>0.12</td>
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<td>540</td>
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<td>1.36 × 10^5</td>
<td>0.35</td>
<td>2900</td>
<td>3.20</td>
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</table>

*Values of monovalent binding properties carried out at 10°C by SPR.

k eff,olig at 10°C estimated from monovalent binding properties and from k eff,olig values at 25°C, using Eq. (2) and the relationship: µ_{eff,C} = µ_{eff,C} × k off,olig/k on,10°C/k off,olig/k on,25°C.
Table IV. Equilibrium constants (K_D) for 2C TCR binding the SIY/K^b (pMHC) complex measured under various conditions

<table>
<thead>
<tr>
<th>Assay Conditions</th>
<th>K_D (µM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble, recombinant 2C TCR and SIY/K^b</td>
<td>32</td>
<td>23</td>
</tr>
<tr>
<td>SPR</td>
<td>27</td>
<td>24</td>
</tr>
<tr>
<td>Solvent monomeric SIY/K^b</td>
<td>0.1</td>
<td>54</td>
</tr>
<tr>
<td>Inhibition of [125I]-Fab 1B2 binding to CD8^+ T cells</td>
<td>0.33</td>
<td>53</td>
</tr>
<tr>
<td>Dimeric SIY/K^b-IgG binding to Naive 2C transgenic T cells</td>
<td>0.004 (4°C)</td>
<td>15</td>
</tr>
<tr>
<td>Activated 2C transgenic T cells</td>
<td>0.077 (37°C)</td>
<td>15</td>
</tr>
<tr>
<td>Streptavidin-oligomers of SIY/K^b binding to CD8^- 2C T cell hybridomas</td>
<td>0.0011</td>
<td>19</td>
</tr>
<tr>
<td>CD8^+ 2C T cell hybridomas</td>
<td>0.0041</td>
<td></td>
</tr>
<tr>
<td>CD8^- m33 T cell hybridomas</td>
<td>0.0003</td>
<td></td>
</tr>
<tr>
<td>CD8^- m33 T cell hybridomas</td>
<td>0.0010</td>
<td></td>
</tr>
<tr>
<td>CD8^- 2C transgenic splenic T cells</td>
<td>0.0016</td>
<td></td>
</tr>
<tr>
<td>CD8^- 2C transgenic splenic T cells</td>
<td>0.0016</td>
<td></td>
</tr>
</tbody>
</table>

Measurements at 25°C unless otherwise indicated.

Values measured in current work.

leads to half-saturation of that TCR subset (Eq. 5), the main uncertainty being the maximum level. From the titration shown in Fig. 1C, K_D, olig is ~1 nM for the 2C TCR–SIY/K^b oligomer interaction on CD8^+ T cells. This value is compared in Table IV with several others measured for the same TCR (2C) and the same Ag (SIY/K^b complex) under different conditions. The values range from ~30 µM (in the micromolar range commonly found by SPR for many rTCR and pMHC pairs) to a 300-fold higher affinity for the binding of soluble SIY/K^b monomer to the 2C TCR and CD8 on intact 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 nonclustered 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 CD8β in the trimeric CDS8–pMHC–TCR reaction. Experimental findings (32) and computational analysis (46) concur in showing that the CDS8 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 trimeric, the CD8 effect can be increased up to 8-fold because k_{off}^{eff} is proportional to (k_{off})^3 (Eq. 2 and Refs. 32, 46). The CD8 effect on hybridomas that expressed the high-affinity TCR (m33) is consistent with these values: t_{1/2} is ~1.4-fold greater for the CD8^pos than CD8^neg cells (Fig. 3A). However, for hybridomas that express the lower affinity 2C TCR, t_{1/2} 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^neg hybridoma and CD8^pos splenic T cells that express the same TCR (2C) (t_{1/2} ~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 k_{off}^{eff} to k_{off}^{-3} (Eq. 2).

Appendix A: Model Equations

Dissociation of bound oligomers

L = T \frac{3k_{off}}{k_{on}} \Rightarrow L = T \frac{2k_{off}}{2k_{on}} \Rightarrow L - T \Rightarrow 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 µ (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 µ correspond to the number of pMHCs available for binding/unbinding.

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

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

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

and

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

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:

$$\frac{dL_B}{dt} = -k_{off}^{eff} L_B = -k_{off}[L - T]. \quad (A5)$$

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:

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

$$[L = T] = \frac{\mu}{3k_{off}^{eff}}[L - T] = \frac{\mu^2}{3k_{off}^{eff}}[L - T]. \quad (A7)$$

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

$$L_B = [L - T] \left(1 + \frac{\mu}{k_{off}^{eff}} + \frac{\mu^2}{3k_{off}^{eff}}\right) = [L - T] \left(\frac{3k_{off}^{eff} + 3k_{off}^{eff} \mu + \mu^2}{k_{off}^{eff}}\right). \quad (A8)$$

Plugging (A8) in to (A5) yields Text Eq. 2:

$$\frac{L_{bound}}{L_{bound,0}} = \exp\left[-k_{off}^{eff} t\right] = \exp\left[-\frac{k_{off}^{eff}}{3k_{off}^{eff} + 3k_{off}^{eff} \mu + \mu^2}\right] \approx \exp\left[-k_{off}^{eff} \frac{\mu^2}{\mu^2 t}\right],$$

where the approximate equality can be applied under the conditions where \(\mu \gg k_{off}\) 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:

$$k_{on}^{eff} T_{free} L_{sol} = k_{off}^{eff} L_{bound},$$

where \(k_{on}^{eff}\) 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:

$$\frac{\theta}{1 - \theta} = \frac{k_{on}^{eff} L_{sol} (3k_{off}^{eff} + 3k_{off}^{eff} \mu + \mu^2)}{k_{off}^{eff}} = \frac{k_{on}^{eff} L_{sol}}{k_{off}^{eff}}.$$

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 cells. For ligand–protein interactions, it is usually expressed as \(r/n - r = K_c\), 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, \(k_{off}^{eff}\) 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**


