Different T Cell Receptor Affinity Thresholds and CD8 Coreceptor Dependence Govern Cytotoxic T Lymphocyte Activation and Tetramer Binding Properties

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T cells have evolved a unique system of ligand recognition involving an antigen T cell receptor (TCR) and a coreceptor that integrate stimuli provided by the engagement of peptide-major histocompatibility complex (pMHC) antigens. Here, we use altered pMHC class I (pMHCI) molecules with impaired CD8 binding (CD8-null) to quantify the contribution of coreceptor extracellular binding to (i) the engagement of soluble tetrameric pMHCI molecules, (ii) the kinetics of TCR/pMHCI interactions on live cytotoxic T lymphocytes (CTLs), and (iii) the activation of CTLs by cell-surface antigenic determinants. Our data indicate that the CD8 coreceptor substantially enhances binding efficiency at suboptimal TCR/pMHCI affinities through effects on both association and dissociation rates. Interestingly, coreceptor requirements for efficient tetramer labeling of CTLs or for CTL activation by determinants displayed on the cell surface operate in different TCR/pMHCI affinity ranges. Wild-type and CD8-null pMHCI tetramers required monomeric affinities for cognate TCRs of $K_D < \sim 80 \mu M$ and $\sim 35 \mu M$, respectively, to label human CTLs at 37 °C. In contrast, activation by cellular pMHCI molecules was strictly dependent on CD8 binding only for TCR/pMHCI interactions with $K_D$ values $> 200 \mu M$. Altogether, our data provide information on the binding interplay between CD8 and the TCR and support a model of CTL activation in which the extent of coreceptor dependence is inversely correlated to TCR/pMHCI affinity. In addition, the results reported here define the range of TCR/pMHCI affinities required for the detection of antigen-specific CTLs by flow cytometry.

In concert with the T cell receptor (TCR),2 the coreceptors CD4 and CD8 participate in and enhance the process of antigen recognition by T cells through extracellular interactions with peptide-major histocompatibility complex (pMHC) molecules (1–3) and amplification of ensuing signal transduction events (4–8). CD8 molecules are predominantly expressed as $\alpha\beta$ heterodimers on the surface of cytotoxic T lymphocytes (CTLs) (9), but are also found in $\alpha\alpha$ homodimeric form on intraepithelial $\alpha\beta$ T lymphocytes, certain subsets of circulating activated CTLs, and the membranes of distinct cell lineages such as $\gamma\delta$ T cells, natural killer T cells, and dendritic cells (reviewed in Ref. 10). CD8$\alpha\alpha$ and CD8$\alpha\beta$ bind directly to invariant domains of major histocompatibility complex class I (MHC) molecules (11–13). Although CD8$\alpha\alpha$ and CD8$\alpha\beta$ bind MHC molecules with similar affinities (14), it is well established that CD8$\alpha\alpha$ is a much poorer coreceptor for CTLs than is CD8$\alpha\beta$. Indeed, an emerging concept is that CD8$\alpha\alpha$ acts as an inhibitor of CTL activation (10). More generally, recent experimental evidence has lent credence to the hypothesis that efficient regulation of CTL activity is mediated by modifications of CD8 coreceptor functions in vivo. These modifications include switching to expression of the CD8$\alpha\alpha$ homodimer, post-translational changes of CD8$\alpha\beta$ following activation (15, 16), and down-regulation of CD8 expression on the cell surface (17–20). Fluctuations in the partitioning and relative distribution of TCR and CD8 molecules on the membrane have also been proposed to influence the interplay between the antigen receptor and coreceptor in the initiating steps of CTL activation. Notably, Pecht and Gakamsky (21) proposed a model in which pre-existing TCR-CD8 complexes constitutively formed on the surface of non-activated CTLs are crucial for the initiation of CTL activation. This hypothesis stems from observations that blocking CD8 engagement and disrupting T cell membrane microdomain organization substantially decrease the binding efficiency and association kinetics of multimeric peptide-major histocompatibility complex class I (pMHCI) molecules (22). This model, together with studies reporting that variations in the glycosylation state and expression levels of CD8 following in vivo antigen encounter affect the binding of pMHCI multimers to CTLs (19, 20), suggests that inhibition of the extracellular engagement of pMHCI by CD8 results in reduced CTL activation.

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The recognition efficiency of all syngeneic pMHCⅠ epitopes is improved by CD8 coreceptor activities. However, the absence of the coreceptor from the cell surface or abrogation of its engagement with MHC molecules does not inhibit T cell activation by different agonist ligands to the same extent (23–26). Activation of a T cell by different epitope variants is thus said to differ in its degree of coreceptor dependence. Two principal, mutually nonexclusive explanations for this phenomenon have been proposed in the CD8 system. First, data obtained by Holler and Kranz (27) and others (23, 25, 28) strongly suggest that coreceptor dependence is inversely correlated with TCR/pMHCⅠ affinity. In this model, CTL activation by weak agonist ligands characterized by low affinity and short half-life interactions with the TCR relies heavily on CD8 coreceptor activity. Second, it has been proposed that positioning of the TCR variable domains (Vα in particular) on the pMHCⅠ platform and the resulting overall conformation of the TCRαβ-CD3 complex may hamper the signaling activity of CD8 (29) and compromise its ability to engage MHCⅠ molecules through binding solutions offering unfavorable positioning of the TCR/pMHCⅠ/CD8 trimolecular complex (30). Therefore, in this model, coreceptor enhancement of CTL activation depends on the geometry of TCR engagement.

In this study, we describe a system that enabled us to examine the effects of the pMHCⅠ/CD8 interaction on the engagement of antigenic ligands by CTLs as a function of TCR/pMHCⅠ affinity. Comparison of the influence of CD8 on the binding efficiency of various soluble pMHCⅠ complexes and on the corresponding cellular activation profiles elicited by these ligands presented on the surface of antigen-presenting cells provides insights into the role of the coreceptor extracellular binding properties in CTL activation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The CTL clone ILA1, isolated from a healthy donor, is specific for residues 540–548 (ILAKFLHWL) of the catalytic subunit of the ubiquitous tumor-associated antigen human telomerase reverse transcriptase (hTERT) presented in association with human leukocyte antigen (HLA) A*0201 (HLA A2 from hereon). Peripheral blood mononuclear cells were stimulated by autologous antigenic presentation using the hTERT-(540–548) peptide at 10 nM in the presence of interleukin-7. Three days after initial antigen exposure, interleukin-2 was gradually added to the culture up to 100 units/ml. Similar rounds of re-stimulation were repeated three times every 12–14 days. Following successful expansion, an antigen-specific T cell line was sorted on the basis of the expression of the activation markers CD25 and CD69 after incubation with the ILAKFLHWL peptide using a FACSVantage (BD Biosciences). The monoclonal T cell line ILA1 was produced by limiting dilution of this enriched line. T cells were initially grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (R10) with 10% T-STEM (final volume; BD Biosciences) and 100 units/ml interleukin-2 and containing mixed irradiated allogeneic feeders from three unrelated donors. The general methods employed for the generation and maintenance of the other CTL lines used in this study were described previously (31). Hmy2.C1R transfectant cells expressing HLA A2 were used in all functional assays (32). These cells were maintained in R10.

**Interferon-γ (IFN-γ) ELISpot**—Antigen-presenting cells (50,000/well; Hmy2.C1R transfectants) in 100 μl of R10 were added to ELISpot plates (Millipore Corp.) coated with anti-human IFN-γ monoclonal antibody (Mabtech AB). CTLs were then added in 100 μl of R10. Peptides were mixed with the cells at the indicated final concentrations. After incubation at 37 °C for 4 h, the plates were washed six times with phosphate-buffered saline, and a secondary biotinylated anti-human IFN-γ monoclonal antibody (D1K, Mabtech) was added for 90 min. The plates were then washed again, incubated with alkaline phosphatase-conjugated streptavidin, and developed with a colorimetric reagent according to the manufacturer’s instructions. Spots were counted using an automated ELISpot reader (Autoimmun Diagnostika GmbH).

**Degranulation Assays**—These assays were carried out as described previously (33). 10,000 CTLs were mixed with 50,000 antigen-presenting cells in the presence of the indicated final peptide concentrations.

**Measurement of TCR Down-regulation**—10,000 antigen-presenting cells were prepulsed with the indicated concentrations of peptide and washed twice with serum-free RPMI 1640 medium supplemented with penicillin, streptomycin, and l-glutamine as above. 30,000 CTLs were added in each assay well and incubated for 4 h at 37 °C in 96-well plates. Cells were pelleted by centrifugation and stained with phycoerythrin-conjugated anti-CD3 and allophycocyanin-conjugated anti-CD8 monoclonal antibodies prior to flow cytometric analysis. Analysis was performed with a FACScalibur flow cytometer (BD Biosciences) using CellQuest software.

**Peptides**—Synthetic peptide preparations of hTERT-(865–873), hTERT-(540–548), and monosubstituted analogs were purchased from Pepscan Systems (Lelystad, The Netherlands). Human immunodeficiency virus-1 (HIV-1) Gag p17 (77–85) (SLYNTVATL) and human T cell lymphotrophic virus-1 Tax (11–19) (LFGYPVVY) peptides were purchased from Invitrogen. The preparations used in this study were purified by mass spectrometry by the manufacturer and showed purities >95%. Powder was initially dissolved in Me2SO and further diluted in serum-free RPMI 1640 medium to the desired concentrations.

**Protein Synthesis**—Soluble biotinylated monomeric pMHCⅠ proteins were produced as described previously (34). Multimerization was performed by the addition of R-phycoerythrin-labeled streptavidin (Molecular Probes) to aliquots to a total pMHCⅠ/streptavidin molar ratio of 4:1. Expression, refolding, purification, and biotinylation of soluble TCR heterodimers were conducted as described previously (35).

**Surface Plasmon Resonance**—A Biacore 3000 TM machine (Biacore, Uppsala, Sweden) and CM-5 sensor chips were used. Approximately 5000 response units of streptavidin were covalently linked to the chip surface in all four flow cells using the amino coupling kit according to the manufacturer’s instructions. Biotinylated pMHCⅠ proteins and biotinylated control
protein (HLA A2 SCa1, HLA A2 MelanA, or HLA A2(D227K/T228A) MelanA) were bound to the sensor surfaces by flowing dilute solutions (50 μg/ml) of protein over the relevant streptavidin-coated flow cell. Approximately 1000 response units of protein ligand were bound to each flow cell for equilibrium affinity measurements. The soluble ILA1 TCR or CD8αα homodimer was then allowed to flow over the relevant flow cells at a rate of 5 μl/min at the concentrations indicated in the figures. All measurements were performed at 25 °C using HEPES-buffered saline (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% Surfactant P20). Responses were recorded in real time and analyzed using BIAevaluation software (Biacore). Equilibrium dissociation constants (Kd) were determined assuming a 1:1 interaction (A + B ⇄ AB) by plotting specific equilibrium binding responses against protein concentrations, followed by nonlinear least-squares fitting of the Langmuir binding equation: 

\[
\frac{[A][B]}{K_d} = \frac{[AB]}{1 + [A][B]}
\]

Conformation of the data to the Langmuir equation was confirmed by linear Scatchard plot analysis using Origin 6.0 software (MicroCal, Northampton, MA). The kinetics of TCR/pMHCI interactions were measured using sensor chips coated with 500 response units of ligand. Analyte TCR was flowed over the chip at a flow rate of 50 μl/min. First-order exponential association (k+a) and dissociation (k−a) curves were fitted simultaneously by nonlinear least squares.

pMHCI Tetramer Staining and Association and Dissociation Measurements—Staining of the various CTL lines and of the ILA1 CTL clone with tetramers was performed at a final concentration of 220 nM (with respect to the monomeric pMHCI component) unless stated otherwise. Titration staining experiments performed at 37 °C with cognate and non-cognate wild-type (WT) tetramers for clones ILA1 and 003 indicated that, with the tetramer preparations used in this study, non-cognate background staining remained negligible for concentrations ≤220 nM (data not shown). This concentration was thus chosen to perform standard CTL tetramer labeling. The staining conditions were 37 °C for 15 min or 4 °C for 45 min. For tetramer association, 10⁶ ILA1 CTLs were washed and resuspended in 150 μl of phosphate-buffered saline; pMHCI tetramers were added at a final concentration of 1 μg/ml (22 nM) with respect to monomer at t₀. Aliquots of 10 μl of each sample were then taken at the indicated time points and diluted to a final volume of 500 μl in phosphate-buffered saline prior to flow cytometric analysis. Tetramer concentrations were thus diluted 50-fold so that further staining occurring after collection did not contribute significantly to the measured mean fluorescence intensity (MFI). Background staining obtained by labeling ILA1 CTLs with non-cognate HLA A2 tetramers for 30 min was subtracted from the MFI value at each time point. All stainings for the association kinetics were performed at room temperature. Detailed procedures for the tetramer decay assay are described elsewhere (36). Staining of ILA1 cells was performed with tetramer concentrations of 2.2 nM for 3G WT HLA A2 and HLA A2(D227K/T228A) tetramers, 8.8 nM for 8Y and 8T WT HLA A2 tetramers, and 220 nM for 8Y and 8T HLA A2(D227K/T228A) tetramers. Unless otherwise stated, CTL labeling was performed at 37 °C in azide buffer (phosphate-buffered saline, 0.1% NaN₃, and 0.5% fetal calf serum). t₀ of the decay assay refers to the addition of anti-HLA A2 monoclonal antibody (clone BB7.2, Serotec).

Tetramer Binding Kinetics and a Model for pMHCI Tetramer Staining—The model is based on the following assumptions. (i) A pMHCI tetramer approaching the cell surface from the incubation solution will initially engage a single TCR molecule. (ii) Subsequent TCR molecules are recruited to this “singlet” cluster by diffusing into the interaction radius of one of the tetramer binding sites and engaging that site, thus forming duplet and triplet clusters. (iii) Binding and rebinding to one of the three available tetramer binding sites occur very rapidly, so loss of temporarily unbound TCR molecules (by diffusion away from the tetramer domain of interaction) is negligible compared with loss by tetramer becoming unbound at all of its sites. (iv) Once a tetramer has become unbound, it will diffuse into solution, whereupon the TCR cluster is left with sufficient time to disband and diffuse into the background of free TCR molecules before the next tetramer binds one of these singlets from solution. (v) Within a triplet cluster, the transition from univalently bound tetramer to bivalently bound tetramer occurs at rate 6µ, where µ is the single-site TCR/pMHCI binding rate (three tetramer sites times two TCRs); the transition from bivalently bound tetramer to trivally bound tetramer occurs at rate 2µ (as a single tetramer site remains available); the transition back from trivally bound tetramer to bivalently bound tetramer occurs at rate 3ν (three bonds), where ν is the single-site dissociation rate; the transition from bivalently bound tetramer to univalently bound tetramer occurs at rate 2ν; and the transition from univalently bound tetramer to unbound tetramer, which subsequently diffuses away into the solution while the TCR triplet dissolves, occurs at rate ν. The fraction of TCR triplets with n-valently bound tetramer (n = 1, 2, 3) is given by a quasi-stationary distribution that can be derived from the consideration of detailed balance equations based on the transition rates. For n = 1, this quasi-stationary value is approximately (ν(ν/µ)²/2, under the assumption that µ >> ν. This means that triplet clusters disappear at a specific rate of ν(ν/µ)²/2. A similar argument can be developed for duplet clusters, giving the fraction (ν(ν/µ)²/3 for univalently bound tetramers, which yields the formula ν(ν/µ)²/3 for the corresponding specific duplet destruction rate. The univalent fraction for singlet clusters is 1, with destruction rate ν. Let Rₙ denote the density of clusters on the T cell surface where n denotes the status of the cluster (n = 0, 1, 2, 3; 0 is a “free” TCR molecule). Conservation of TCR molecules on the cell surface implies the following (Equation 1),

\[
R_T = R_0 + R_1 + 2R_2 + 3R_3
\]

(Eq 1)

where Rₜ is the total density. The intensity of the stain (I) is assumed to be proportional to the sum R₁ + R₂ + R₃, which can be evaluated by calculating these values for a dynamic equilibrium between the various types of cluster. Singlet clusters form at rate ψₙRₙ, where ψ is the rate at which free TCR molecules capture a tetramer from solution. (This rate is proportional to the concentration of tetramer in the medium.) Singlet clusters break up at rate ν and recruit free TCR molecules at θνRₙ. Duplet clusters break up at a rate of ν(ν/µ)²/3 and recruit free
Coreceptor Dependence of CTL Activation and Tetramer Binding

TCR molecules at $\theta_2 R_\sigma$. Triplet clusters break up at a rate of $\nu (\nu/\mu)^2/2$ and are incapable of recruiting additional TCR molecules. Setting creation and destruction rates equal for each of these species and assuming (i) that rates $\mu$, $\psi$, $\theta_1$, and $\theta_2$ are each proportional to the TCR/pMHCI on-rate, whereas rate $\nu$ is proportional to the TCR/pMHCI off-rate, and (ii) that the TCR recruitment rates are negligible in comparison with the break-up rates, we obtain two equations. First, the TCR conservation law becomes

$$1 = r_0 + (K_1/K_0)r_0 + 2(K_2/K_0)^2 r_0^2 + 3(K_3/K_0)^3 r_0^3$$  \hspace{1cm} (Eq. 2)

where $r_0 = R_0/R_1$; $K_3$ is the TCR/pMHCI dissociation constant; and $K_1$, $K_2$, and $K_3$ are compound parameters absorbing the assumed proportionality relationships with the on-rates and off-rates. Given $K_3$, the conservation law can be solved for $r_0$, and the relative intensity of the tetramer stain can then be calculated from Equation 3.

$$(R_3 + R_2 + R_1)/R_7 = r_0 + (K_1/K_0)r_0 + (K_2/K_0)^2 r_0^2 + (K_3/K_0)^3 r_0^3$$

(Eq. 3)

Maximum staining intensity is attained when all TCR molecules are bound at a 1:1 stoichiometry to tetramers, i.e. when all TCR molecules are bound in triplets ($R_3 = R_7$). This does not correspond to the case in which $K_3$ becomes vanishingly small; in that case, all TCR molecules are bound in triplets ($R_3 = R_7$), and the relative staining intensity is only one-third of the maximum. For data fitting, a standard least-squares procedure was employed, with minimization of the sum of squares by means of steepest descent. Equations 2 and 3 were solved by bisection. Data were log-transformed prior to the formation of the sum of squares, corresponding to a log-normal assumption on measurement noise and the standard procedure for homogenizing the variance. Estimates were expressed as the value that minimized the sum of squares $\pm$ S.D., which is the square root of the estimated parameter variance, being the inverse second derivative of the sum of squares relative to the parameter, evaluated at the point estimate, times the error (noise variance) estimated by the sum of squares.

According to the kinetic assumptions listed above, the establishment of pMHCI tetramer equilibrium staining is described by a complex nonlinear six-dimensional dynamic system. The staining intensity ($I$) is described by the following ordinary differential equation (Equation 4),

$$\dot{I} = \psi R_7 - \lambda_{\text{eff}}(t) I$$

(Eq. 4)

where $\lambda_{\text{eff}}(t)$ is a time-varying effective rate constant, which is a well defined function of the six-dimensional state of the system. Empirically, it is found that the association kinetics are an excellent fit to the biphasic exponential model

$$I(t) = I_{\text{max,fast}}(1 - \exp(-\lambda_{\text{fast}} t)) + I_{\text{max,slow}}(1 - \exp(-\lambda_{\text{slow}} t))$$

(Eq. 5)

(see Fig. 6, A and B). The parameters of this equation were determined by nonlinear least squares. It follows from Equation 5 that $\lambda_{\text{eff}}$ relaxes to steady-state value $\lambda_{\text{eff}}(\infty)$, which can be calculated from the parameter estimates according to the formula

$$(I_{\text{max,fast}} + I_{\text{max,slow},\text{slow}})/(I_{\text{max,fast}} + I_{\text{max,slow}}).$$

**RESULTS**

Diverse pMHCI Binding Patterns Reflect Distinct Recognition Properties in Polyclonal and Monoclonal CTL Populations—Various CTL lines specific for HLA A2-restricted viral (HIV-1 Gag p17-(77–85) and human T cell lymphotropic virus-1 Tax-(11–19)) or tumor-derived (hTERT-(865–873)) peptides were established from antigen-experienced or antigen-naive individuals and stained with cognate WT or CD8-null pMHCI tetramers (Fig. 1, A and B); the latter were constructed from HLA A2 monomers containing a double D227K/T228A mutation in the $\alpha_3$ domain, which abrogates binding to the CD8 coreceptor (32). In CTL lines derived from antigen-experienced individuals, both WT and CD8-null pMHCI tetramers identified cognate T cell populations of similar magnitude. However, the CD8-null tetramers consistently stained with lower MFI values compared with the WT tetramers at identical concentrations (Fig. 1, A and B). In contrast, a line specific for human T cell lymphotropic virus-1 Tax-(11–19) derived from an antigen-naive individual (SH) stained only with the corresponding WT pMHCI tetramer. Furthermore, antigen-responsive CTLs expanded from a naïve background by successive autologous stimulations with the hTERT-(865–873) peptide failed to bind either WT or CD8-null pMHCI tetramers at any concentration (Fig. 1A) (data not shown), yet produced IFN-γ when exposed to cognate peptide (Fig. 1C). These data indicate that functional CTL populations can exhibit discrete tetramer binding profiles and CD8 dependencies. Similar observations applied to different CTL clones (Fig. 1D) (data not shown). These distinct patterns of pMHCI tetramer binding correlated with the efficiency of antigen recognition in IFN-γ release assays (Fig. 1E). Overall, these results indicate that CTLs with high levels of functional avidity can be selectively identified with CD8-null pMHCI tetramers, as reported previously (37–39), whereas functional CTLs with low antigen sensitivity fail to bind WT or CD8-null pMHCI tetramers.

Functional and Biophysical Characterization of hTERT-(540–548) Altered Peptide Ligands with Different Stimulatory Activities—Systematic screening of a library of monosubstituted peptide variants using the ILA1 CTL clone enabled us to identify an array of ligands that elicited distinct functional outcomes in cellular activation assays (data not shown). Two weak agonist, three superagonist, and an antagonist peptide were selected for further study (Fig. 2 and Table 1). The affinity of the ILA1 TCR for these various ligands was measured in surface plasmon resonance (SPR) binding equilibrium experiments. The measured dissociation constants spanned a wide spectrum of values (Fig. 3 and Table 1), from $K_D$ values of 1 μM (close to the highest measured syngeneic interaction affinities) for 3G and 3G8T variants to high $K_D$ values corresponding to very weak interactions (242 μM for 5Y). ILA1 TCR binding to the 8E weak agonist was so weak that we were unable to determine a reliable $K_D$ from SPR binding equilibrium experiments (Fig. 3H). Interestingly, the non-stimulatory antagonist variant 7Y showed an affinity for the TCR substantially superior to the
weak agonist peptides 5Y and 8E (Fig. 3 and Table 1). There was an overall correlation between the potency of the various agonist ligands and their affinity for the ILA1 TCR, with the notable exception of the two high affinity superagonists 3G and 3G8T. Despite 

\[ K_D \]

values almost 10-fold lower than those measured for the two best superagonists (8Y and 8T), these two variants were less stimulatory in all cellular activation assays (Fig. 2 and Table 1) (data not shown). These results further serve to highlight that TCR/pMHC affinity is not the main correlate of ligand potency for cellular activation. Partial analysis of ILA1 agonist pMHCI kinetic parameters was carried out to determine whether these ligands complied with the kinetic proofreading model of T cell activation and whether the high affinity for the ILA1 TCR of the relatively weak potency ligands 3G and 3G8T could be explained by unusual kinetic features such as very fast on-rates. The association rate constant \( k_{on} \) for the 3G antigen was indeed substantially faster than those for the other ligands studied (Fig. 4 and Table 1). However, for the 3G altered peptide ligand, the dissociation rate constant \( k_{off} \) for the TCR/pMHCI interaction was twice as slow compared with the most potent agonist, 8T (Table 1). Thus, the low potency of 3G compared with 8T could not be explained solely by the kinetic proofreading model. The kinetic parameters of the interaction between the ILA1 TCR and the weakest agonists 5Y and 8E were too fast for reliable measurement (Fig. 4). Nevertheless, it was apparent that the ILA1 TCR exhibited specific binding to both of these ligands. Comparison of the response unit traces

![Figure 1](http://www.jbc.org/content/282/33/23803.full)
for the 5Y and 8E ligands (Fig. 4, E and F) confirmed that 8E was a much poorer ligand than 5Y. Although it is not possible to give an accurate affinity for binding to the 8E variant from these experiments, it is clear that the $K_D$ was $>500 \mu M$.

**Tetramer Staining and TCR/pMHCI Interaction Affinity—** The intensity or brightness of T cell labeling using pMHC tetramers is generally thought to be an indicator of functional avidity and efficiency of antigen recognition in both MHCI (40, 41) and MHCI (42) systems. In the case of pMHCI tetramers, invariant binding of the CD8 coreceptor is also known to influence staining intensity. We used the ILA1 system to study the efficiency of pMHCI tetramer binding in relation to the affinity of the monomeric TCR/pMHCI interaction. At 37 °C, ILA1 CTLs stained at similar intensities with WT tetramers for which the monomeric pMHCI complex exceeded an affinity threshold for the TCR ($K_D < 25 \mu M$); progressive decreases in tetramer binding were observed at higher $K_D$ values (Fig. 5, A and C). Similar results were obtained with the corresponding CD8-null tetramers, although the drop-off set in at substantially higher affinities ($K_D < 10 \mu M$) (Fig. 5, B and C). Staining was even more stringent when performed at 4 °C (Fig. 5D). In each case, the drop-off was sharp (occurring within about half a decade).

This can be understood on the basis of tetramer binding kinetics. The monomeric $K_D$ contributes to both the formation and persistence of a tetrameric bond to a TCR triplet cluster on the cell surface, yielding six multiplicative steps in the kinetics (see “Experimental Procedures” for details). Fitting a mathematical model to these kinetics indicated that the pMHCI/CD8 interaction prolongs the average monomeric TCR/pMHCI dwell time by a factor $\sim 2.3$, in keeping with earlier estimates based on tetramer dissociation experiments (36).

**Coreceptor Engagement and Soluble pMHCI Association and Dissociation Kinetics—** A recent study documented that blocking the engagement between MHCI molecules and the CD8 coreceptor slows pMHCI tetramer association at the cell surface (22). As anti-CD8 antibodies seem to have a range of effects on pMHCI tetramer binding that do not reflect only disruption of the pMHCI/CD8 interaction (43, 44), we sought to reproduce this observation in the ILA1 system using CD8-null pMHCI tetramers. Three pMHCI ligands that showed significant staining in tetrameric form in the absence of CD8 binding (3G, 8T, and 8Y) (Fig. 5B) were selected for real-time binding analysis (Fig. 6, A and B). Data fitting showed a marked difference in the effective association rates between WT and CD8-
FIGURE 3. Affinity measurements of the interaction between the ILA1 TCR and various cognate pMHCI ligands. The results from nonlinear analysis of SPR binding equilibrium experiments using soluble TCRs at a maximum concentration of 100 or 300 μM and 2-fold dilutions thereof flowed over immobilized pMHCI complexes are shown here. $K_D$ values were determined by analyzing the data in nonlinear curve fittings to the equation $AB = B \times AB_{max}/(K_D + B)$ assuming 1:1 Langmuir binding. Mean $K_D$ values and the corresponding S.D. values are shown for each ligand. The integrity of pMHCI proteins was verified by examining CD8α binding using SPR (data not shown). Binding to the 8E variant was so weak that it failed to approach equilibrium even with the highest concentration of TCR utilized. Our estimates from both equilibrium binding and kinetic (Fig. 4) experiments with this ligand suggest a binding $K_D$ of $\approx 2 \text{mM}$. However, these estimates are potentially subject to substantial errors. Comparison with experiments with the 5Y ligand (also see Fig. 4) confirmed that the ILA1 TCR interaction with the 8E variant ligand must be extremely weak ($K_D > 500 \text{ μM}$).

FIGURE 4. Kinetic measurements of ILA1 TCR interactions with five different agonist pMHCI ligands. Upper panels, SPR kinetic measurements were performed with five serial 2-fold dilutions of 50 μM ILA1 TCR for the index (A), 3G (B), and 8T (C) variants. The plots show data and curve fitting from two separate experiments. Similar results (±5%) were obtained from three separate experiments with these protein preparations and from three other experiments using separately prepared pMHCI and TCR preparations. Lower panels, 10 serial dilutions at a higher TCR concentration (300 μM) were used in an attempt to measure the binding with weak agonists ligands. Data obtained with the index ligand (D) were comparable with those obtained with the lower concentration (A) of separately prepared TCR and pMHCI. However, the kinetics were too fast for accurate measurement for the 8E (E) and 5Y (F) ligands. All pMHCI ligands bound equally well to the CD8α protein (data not shown).
null pMHCI tetramers, consistent with observations by Gakowsky et al. (22). Calculation of $\lambda_{\text{off}}(\infty)$ for tetramer staining kinetics (see “Experimental Procedures”) revealed that abrogation of the pMHCI/CD8 interaction substantially reduced the capture rate of soluble pMHCI tetramers by ILA1 CTLs. This reduction was estimated to be 61% for 3G, 85% for 8Y, and 90% for 8T.

The stabilizing effect of the coreceptor was also assessed in the ILA1 system using pMHCI tetramer decay assays (Fig. 6, C and D). In the case of 8T and 8Y altered peptide ligands, increasing the concentration of CD8-null tetramers did not result in staining intensity values similar to those obtained with WT tetramers at 220 nm (data not shown); to achieve similar MFI values for stainings with both types of tetramer, the concentrations of the WT HLA A2 tetramers were adjusted. As reported previously (36), abrogation of the pMHCI/CD8 interaction markedly enhanced the tetrameric pMHCI dissociation rate. All three selected pMHCI complexes (3G, 8T, and 8Y) displayed similar dissociation patterns regardless of their respective affinities for the TCR. The interaction half-lives and dissociation kinetics for all three ligands in WT pMHCI tetrameric form were nearly identical (Fig. 6C); the same held true for these ligands in CD8-null pMHCI tetrameric form, although in each case, the $t_{1/2}$ values were approximately three times smaller. The increased avidity afforded by ligand multimerization thus seemed to have similar consequences on the stability of each complex regardless of the affinity of the corre-

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FIGURE 5. Staining of ILA1 CTLs with tetramerized cognate pMHCI molecules. Shown is the staining of CTL clone ILA1 with seven different hTERT-(540–548) variants, as indicated, refolded with WT HLA A2 (A) or CD8-null HLA A2(D227K/T228A) (B) at 37 °C. The MFI values observed with pMHCI tetramer staining are plotted versus the TCR/pMHCI interaction $K_D$ values for experiments conducted at 37 °C (C) and 4 °C (D) with WT HLA A2 (circles) and CD8-null HLA A2 (squares) molecules for each variant added at a final concentration of 220 nm (10 μg/ml). Color codes correspond to those shown in A and B. Staining with the set of altered peptide ligands refolded with each type of heavy chain was performed at least three times. Representative data are shown. Curves are the best fit of the model described under “Experimental Procedures,” with estimates as follows: background staining, 2.32 ± 0.213; CD8 half-life prolongation factor (WT over CD8-null), 2.29 ± 0.08; $K_D^{(WT)}$, 10.4 ± 1.06 μM; $K_D^{(CD8)}$, 27.7 ± 0.86 μM; $K_i$ fixed at 0 μM; and maximum MFI signal fixed at 225.
Coreceptor Dependence of CTL Activation and Tetramer Binding

The experimental system described here enabled us to quantify the contribution made by CD8 invariant binding to cognate ligand engagement by CTLs under standardized conditions. Using soluble multimerized ligands, we have shown that CD8 substantially improves the binding efficiency of cognate pMHCI molecules with intermediate to low affinities for the TCR. The observation that CD8 binding increases the number of stably bound pMHCI complexes can be explained by enhancement of the association and/or reduction of the dissociation rates. The present data support a role for the coreceptor in both phenomena (Fig. 6). From a functional point of view and in light of the kinetic discrimination model of T cell activation, the increase in individual TCR/pMHCI dwell times enabled by CD8 engagement would be expected to make a significant contribution to the well characterized enhancing effect that the coreceptor confers on the sensitivity of antigen recognition (36). In addition, as proposed by Pecht and Gakowsky (21), enhancement of the pMHCI association rate by CD8 may also increase the overall number of productively engaged TCR complexes and thereby enhance the antigenicity of cognate ligands.

The magnitude of the coreceptor effect on the binding efficiency of cognate pMHCI molecules was clearly influenced by monomeric TCR/pMHCI affinities. At saturating concentrations of ligand, abrogation of CD8 engagement substantially reduced binding of tetrameric pMHCI molecules exhibiting affinities for the TCR with $K_D$ values in the range of $10^{-30} \mu M$ (Fig. 5). For TCR/pMHCI interactions with $K_D \approx 30 \mu M$, coreceptor engagement became obligatory for tetramer binding. In contrast, functional assays with the same epitopes presented on soluble multimerized ligands, we have shown that CD8 substantially improves the binding efficiency of cognate pMHCI molecules with intermediate to low affinities for the TCR. The observation that CD8 binding increases the number of stably bound pMHCI complexes can be explained by enhancement of the association and/or reduction of the dissociation rates. The present data support a role for the coreceptor in both phenomena (Fig. 6). From a functional point of view and in light of the kinetic discrimination model of T cell activation, the increase in individual TCR/pMHCI dwell times enabled by CD8 engagement would be expected to make a significant contribution to the well characterized enhancing effect that the coreceptor confers on the sensitivity of antigen recognition (36). In addition, as proposed by Pecht and Gakowsky (21), enhancement of the pMHCI association rate by CD8 may also increase the overall number of productively engaged TCR complexes and thereby enhance the antigenicity of cognate ligands.

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of the different ligands tested showed a slight reduction in potency (Fig. 7 and Table 1). The 5Y variant displayed an affinity for the ILA1 TCR at the low end of previously characterized TCR/pMHCI interactions with a $K_D$ of 242 nM (Fig. 3). Both kinetic parameters and equilibrium binding analysis demonstrated that the ILA1 TCR bound to the 8E variant with an extremely low binding affinity ($K_D < 500$ nM). However, this ligand still elicited substantial activation in functional assays. Interestingly, the antigenicity of both the 5Y and 8E pMHCI complexes was entirely dependent on intact MHCI/coreceptor interactions. This suggests that enhancement of the TCR/pMHCI association rate mediated by CD8 is crucial in determining the antigenicity of low affinity ligands with unusual kinetic features. Thus, although the degree of coreceptor dependence for tetramer binding and CTL activation is similarly dependent on monomeric TCR/pMHCI affinity, there is a quantitative disparity between the binding effects of the coreceptor on soluble pMHCI engagement and on activation by cell-surface determinants. Such a differential contribution of CD8 to tetramer binding and CTL activation had been suggested by the observation that CD8-negative cells bearing a T specific for an HLA A2-restricted hepatitis C virus epitope cannot bind tetramers, but are able to recognize this epitope displayed on the surface of antigen-presenting cells (46).

In apparent contradiction to kinetic proofreading models of T cell activation, the two ligands with the highest affinities for the TCR (Fig. 3) and the longest half-lives (3G and 3G8T) were not the most potent agonists (Figs. 2 and 4 and Table 1) (data not shown). Such inadequate behavior by T cell ligands has been described previously and has prompted researchers to develop and modify the kinetic proofreading concept. Notably, it lead to the integration of the notion of TCR-binding site plasticity, represented by the thermodynamic variable of heat change capacity, as an essential parameter governing T cell activation efficiency (47, 48). Alternatively, Yachi et al. (30) have recently proposed that exceptions to the kinetic proofreading dogma may result from impaired coreceptor recruitment to the vicinity of the TCR-CD3 complex because of unfavorable orientation of the TCR upon binding to pMHCI. The data we report do not support this concept. First, detailed examination of 3G and 3G8T tetramer binding clearly indicated that CD8 contributes to the enhancement of both their association kinetics and the stability of bound complexes (Fig. 6) (data not shown), indicating that coreceptor engagement is not compromised in the case of these two ligands. Second, activation of ILA1 with antigen-presenting cells expressing HLA A2 molecules that cannot engage CD8 does not result in an inversion of the hierarchy of ligand potency between 3G and 8T, as would be predicted by the kinetic proofreading model (Fig. 7 and Table 1). Thus, our results argue in favor of a model

![FIGURE 7. Effect of the CD8 coreceptor on antigen recognition by ILA1 CTLs. A–D, the response of ILA1 to four different agonist ligands was determined in CD107a up-regulation assays. Each agonist peptide was presented by Hmy2.C1R cells expressing either CD8-null HLA A2(D227K/T228A) (○) or WT HLA A2 (■) molecules. Results are expressed as the percentage of effector cells showing significant activation. Means ± S.D. were calculated from two sample replicates and are representative of at least two independent experiments in each case. E–H, IFN-γ secretion induced by peptide ligands presented in the context of WT (■) and CD8-null (○) HLA A2 molecules is expressed as a percentage of the total resting ILA1 CTLs added per sample. Error bars represent the S.D. of two replicate experiments.](http://www.jbc.org/content/journal/jbc/282/33/23808.full.html)
in which the CD8 dependence of cellular activation correlates with the relative affinities of the TCR for pMHCI complexes, as demonstrated by Holler and Kranz (27) in the 2C TCR system. However, the TCR/pMHCI affinity thresholds for CD8 dependence we observed in the human ILA1 system are very different from those proposed by these authors; their data showed that T cell activation is highly coreceptor-dependent for cognate TCR/pMHCI interactions with a $K_D$ exceeding 3 $\mu M$. In our system, a high degree of coreceptor dependence was obvious only for low affinity interactions exhibiting $K_D$ values in excess of 50 $\mu M$ at the very least, even though abrogation of pMHCI/CD8 binding resulted in a marginal decrease in sensitivity for all high affinity ligands (Fig. 7 and Table 1). A fundamental difference in the experimental approach used in both studies might account for this discrepancy. Holler and Kranz used hybridomas transfected or not with CD8$\alpha$ and CD8$\beta$ chains, whereas in our system, the pMHCI/coreceptor interaction was impaired by point mutations in the MHCI molecules. Normal levels of CD8$\alpha$ and CD8$\beta$ were expressed in the CTLs we used, and notably, there was no disruption to coreceptor association with the CD3 complex, the intracellular kinase Lck, or lipid rafts. Thus, our system dissociates the signaling functions and membrane partitioning roles of CD8, believed to be important in CTL activation, from its extracellular engagement of the MHCI molecules. Therefore, a likely explanation for the observed affinity threshold discrepancy is that increases in the association kinetics and stabilization of the TCR/pMHCI interaction afforded by CD8 extracellular binding are the sole phenomena accounting for the results we obtained in the ILA1 system. In contrast, disruption of the synergistic extracellular binding effects and intracellular coreceptor functions of CD8 in the system of Holler and Kranz probably resulted in a higher stringency of coreceptor dependence. A recent study suggested that the signaling properties of CD8 act in synergy with the TCR/pMHCI stabilization effect and are likely to have a dominant effect in the overall enhancement of the antigen sensitivity phenomenon conferred by the coreceptor (49). The difference of coreceptor dependence affinity thresholds observed in our study and in that of Holler and Kranz fits well with this concept. The fact that the murine pMHCI/CD8 interaction can be of $\geq$4 times higher affinity than the equivalent human interaction (32) is also likely to contribute to differences in the role of CD8 binding in the two species.

Overall, our data suggest that the dynamics of CD8 cell-surface expression, membrane segregation, and post-translational regulation known to modulate the state of responsiveness during T cell development alter the modalities of pMHCI binding, with important consequences for the engagement of lower affinity ligands in particular (Figs. 5 and 7). Notably, the differential contributions of pMHCI/CD8 interactions to the binding of soluble cognate ligands and CTL activation suggest that the intracellular coreceptor activities of CD8 exert their effect at least partially independent of extracellular pMHCI/CD8 engagement. Our findings also imply that rare polymorphisms in MHCI molecules that diminish CD8 binding, such as those that occur at position 245 in HLA A68, HLA B48 and HLA B81, alter the influence of coreceptor engagement on CTL activation.

Our demonstration that CD8 substantially improves the binding efficiency of cognate pMHCI molecules with intermediate to low binding affinity for the TCR indicates that CD8 plays an important role in T cell cross-reactivity or “polyspecificity.” It is well established that T cells are able to recognize a very large number of different peptides (reviewed in Ref. 50). Studies with murine hybridomas have shown that CD8 can alter the fine specificity of allogeneic (51) and syngeneic (27) recognition. Indeed, our ongoing studies show that the majority of ligands recognized by CTL cannot be recognized at any peptide concentration in the absence of pMHCI/CD8 engagement. Thus, the CD8 coreceptor may serve to optimize T cell polyspecificity.

From a practical perspective, our data delineate the range of TCR/pMHCI affinities that are amenable to detection with fluorescent avidin-tetramerized pMHCI molecules. Ligands with $K_D$ values $<70–80 \mu M$ could be detected with WT pMHCI tetramers in our HLA A2-restricted system, whereas CD8-null pMHCI tetramers could label only ILA1 CTLs if the monovalent affinity for the TCR was higher ($K_D < 30 \mu M$) (Fig. 5). These results provide biophysical validation of the observations that CD8-null pMHCI tetramers can qualitatively distinguish CTL populations with high functional avidity (37–39) and confirm that staining intensity with tetramers correlates with functional avidity and TCR/pMHCI affinity (40, 41). It should be noted that parameters other than intrinsic avidity, such as variations of TCR density, differences in membrane lipid organization (52), the state of T cell activation (19, 53, 54), and differentiation status, can also influence tetramer binding avidity and are subject to substantial variation. These considerations preclude generalization of the tetramer staining affinity thresholds established in the system described here, in which all of these variables were standardized. Nevertheless, our results reveal the limitations of tetramer technology by providing direct evidence that functionally competent T cells can bear TCRs with affinities for cognate ligands below the threshold required for pMHCI tetramer engagement. Both human and murine “tetramer-negative” functional T cells have been reported previously (55–58). In one such system, Buslepp et al. (58) measured the affinity between the TCR and pMHCI by SPR; the $K_D$ value of $\approx 80 \mu M$ they measured is consistent with our own results. Finally, our data suggest that soluble multimeric pMHCI molecules engineered to bind the coreceptor with enhanced affinities (36) might enable the detection of cognate ligands with extremely low affinities for the TCR, such as might characterize clonotypes specific for tumor-related or autologous antigens.

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Different T Cell Receptor Affinity Thresholds and CD8 Coreceptor Dependence Govern Cytotoxic T Lymphocyte Activation and Tetramer Binding Properties

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