Interaction between the CD8 Coreceptor and Major Histocompatibility Complex Class I Stabilizes T Cell Receptor-Antigen Complexes at the Cell Surface*

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The off-rate ($k_{off}$) of the T cell receptor (TCR)/peptide-major histocompatibility complex class I (pMHCI) interaction, and hence its half-life, is the principal kinetic feature that determines the biological outcome of TCR ligation. However, it is unclear whether the CD8 coreceptor, which binds pMHCI at a distinct site, influences this parameter. Although biophysical studies with soluble proteins show that TCR and CD8 do not bind cooperatively to pMHCI, accumulating evidence suggests that TCR associates with CD8 on the T cell surface. Here, we titrated and quantified the contribution of CD8 to TCR/pMHCI dissociation in membrane-constrained interactions using a panel of engineered pMHCI mutants that retain faithful TCR interactions but exhibit a spectrum of affinities for CD8 of >1,000-fold. Data modeling generates a “stabilization factor” that preferentially increases the predicted TCR triggering rate for low affinity pMHCI ligands, thereby suggesting an important role for CD8 in the phenomenon of T cell cross-reactivity.

CD8$^+$ cytotoxic T lymphocytes (CTL)$^3$ recognize protein antigens in the form of short peptides presented in association with MHCI molecules on the surface of target cells. Antigen specificity is conferred by the TCR, the highly variable complementarity-determining regions of which interact with the peptide-binding platform of the MHC molecule (1, 2). The pMHCI complex also interacts with the T cell surface glycoprotein CD8, which binds to invariant regions of the MHCI molecule (3, 4).

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* The online version of this article (available at www.jbc.org) contains supplemental data on the decay of pMHCI tetramers (Figs. S1 and S2) and on pMHCI/CD8 interaction (Fig. S3).

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1 The abbreviations used are: CTL, CD8$^+$ cytotoxic T lymphocyte; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HIV, human immunodeficiency virus; HTLV, human T cell lymphotropic virus; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; MHCI, MHCI class I; pMHCI, peptide-MHCI; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; SPR, surface plasmon resonance; TCR, T cell receptor.
monomeric interaction is of low affinity and if high concentrations of soluble protein are used (22). In addition, the current view that the extracellular domains of the TCR and CD8 do not cooperate in binding pMHC (12, 13) is supported by structural studies that indicate that the binding of one molecule is unlikely to alter the affinity for the other (3). However, structural and biophysical studies of soluble TCR and CD8 do not rule out the possibility that CD8 on the CTL surface enhances TCR binding to pMHC on the antigen-presenting cell surface. Mounting evidence shows that CD8 can interact directly with the TCR (17, 23–29) and appears to have an important role in the possibility that CD8 on the CTL surface enhances TCR binding to pMHC on the antigen-presenting cell surface. Mounting evidence shows that CD8 can interact directly with the TCR (17, 23–29) and appears to have an important role in


cells maintained. CTL clone EBV-A is specific for the HLA A2-restricted, HIV-1 p17 Gag-derived epitope GLCTLVAML (38), and the CMV-derived pp65 protein epitope NLVP-SLYNTVATL (37), the EBV-derived BMLFI-encoded epitope GLCTLVAML.

Inclusion Body Preparation—Inclusion bodies from monomeric A2 heavy chain was expressed under the control of a T7 promoter as insoluble inclusion bodies in Esherichia coli strain BL21(DE3)/pLyS5 (Novagen). Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to the recombinant expression medium to a final concentration of 10 mM. The recombinant supernatant was harvested by centrifugation, and the inclusion bodies were solubilized in a buffer containing 8 M urea, 1 M NaCl, 0.5 M imidazole (pH 7.4), and 10% glycerol. The solubilized inclusion bodies were purified by using a Ni-NTA agarose column (Qiagen) and eluted with a buffer containing 8 M urea, 1 M NaCl, 0.5 M imidazole (pH 7.4), and 10% glycerol. The purified inclusion bodies were dialyzed against a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% (w/v) NaN3, and 0.1% (v/v) Triton X-100, and the concentration of the sample was determined by absorbance at 280 nm.

Biophysical Validation of Mutant pMHC Affinities for CD8 and TCR—The D227K/T228A mutation in the α3 domain of HLA A2 has been shown previously to abrogate CD8 binding (8); the A245V mutation reduces CD8 binding by >4-fold (16). The biophysical properties of the Q115E α2 domain mutant and the A2/K β hybrid pMHC proteins were determined by SPR using a BIAcore 3000 (BIAcore AB, St. Albans, UK) machine. sCD8α wild type was prepared as described previously (40). The A6 TCR specific for the HLA A*0201-restricted HTLV-1 Tax epitope LLPFGPVVV was refolded as described previously (41). All proteins for analysis were diluted into HBS-EP buffer (BIAcore AB) containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20. A standard amine coupling kit (BIAcore AB) was used to activate the surface of a research grade CMS sensor chip (BIAcore AB). Streptavidin was covalently coupled to the chip surface by primary amines through the injection of a 0.2 mg/ml streptavidin solution (Sigma) diluted in 10 mM sodium acetate (pH 4.5) over the surface. Biotinylated pMHC1 monomers were immobilized onto the chip surface at ~1,000 response units in each flow cell. Serial dilutions of either sCD8α wild type or soluble A6 Tax TCR in HBS-EP buffer were flowed over the chip to generate kinetic data. Data were analyzed using BIAeval, Excel, and Origin version 6.1 (Microcal software). Kd values were calculated both by linear Scatchard plots and non-linear analysis assuming 1:1 Langmuir binding (A + B ⇔ AB) using non-linear curve fitting to the equation AB = B × (AB)max/Kd + B. Each batch of protein was validated by SPR prior to experimentation.

Tetramerization and Flow Cytometry—Fluorescent tetrameric pMHC1 complexes were produced by mixing phycocerythrin-conjugated streptavidin (Molecular Probes or Prozyme Inc.) and biotinylated pMHC1 monomers at a 1:4 molar ratio, respectively. For ex vivo analysis, 105 peripheral blood mononuclear cells (PBMCs) were stained with the wild type and mutant tetramers shown at the indicated concentrations for 20 min at 37 °C, washed once in fluorescence-activated cell sorter (FACS) buffer (PBS without Ca2+/Mg2+, 1% bovine serum albumin (BSA), and 0.1% NaN3), surface-stained with pre-titered allophycocyanin-conjugated anti-CD8 and peridinin chlorophyll protein-conjugated anti-CD3 monoclonal antibodies (BD Biosciences) for 30 min at 4 °C, and then washed twice and fixed in 1% paraformaldehyde. Data were collected using a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar Inc., San Carlos, CA). For the in vitro expanded SLYNTVATL-specific CTL line 868, 2 × 106 CTLs were stained as described above with the indicated pMHC1 tetramers at the concentrations shown for 20 min at 37 °C, washed, and then stained with allophycocyanin-conjugated anti-CD8 monoclonal antibody (clone SKI; BD Biosciences) and 7-amo actinomycin D (ViaProbe; BD Biosciences) for 30 min on ice prior to further washes and data collection; analysis was performed with CellQuest software (BD Biosciences). All pMHC1 tetramers used in this study were made fresh for the week of use from pMHC1 monomers stored at ~80 °C to minimize the effect of stability differences (15). Once prepared, tetramers were stored in the dark at 4 °C.

pMHC Tetramer Decay Assay—Indicated numbers of CTL were stained in 100 μl of azide buffer (PBS, 0.1% NaN3, and 0.5% fetal calf serum) for 20 min on ice with a concentration of tetramer, previously determined by titration, to give a starting mean fluorescence-activation-density (MFU) of 200; 7-amo actinomycin D (ViaProbe, BD Biosciences) was included so that dead cells could be gated out of the analysis. After washing twice in ice-cold azide buffer, CTLs were resuspended in azide buffer, split into two separate aliquots, and placed at room temperature. To one sample, an excess of unconjugated anti-HLA A2 monoclonal antibody (clone BB7.2; Serotec) at 100 μg/ml was added to block tetramer rebinding. Cells were then taken at time points 0, 1, 2, 5, 10, 15, 20, 30, 40, and 60 min, resuspended in PBS, and analyzed on a FACSCalibur flow cytometer.

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before rebinding can occur. If rebinding is slow (relative to \( k_{off} \)) the dissociation curve is sigmoid, with a shape governed by the mixture of unoccupied (free) CD8 molecules on the T cell. Finally, let \( P \) denote the rate at which MHCI and CD8 dissociate. Then we attain Equation 5,

\[
\gamma_P = |CD8|/K_{D,2D}
\]  

(Eq. 5)

where \(|CD8|\) is the surface density of free CD8 molecules in the contact area and \( K_{D,2D} \) is the two-dimensional dissociation constant of the MHCI/CD8 interaction. We now develop a simple mathematical model that describes how the true single-site off-rate \( k_{off} \) is derived from \( k_{off}^s \) and \( k_{off}^a \). Consider a MHCI molecule that engages a TCR molecule at time \( t = 0 \). Let \( P(t) \) denote the probability that dissociation of this TCR/pMHCI does not occur before time \( t \) while the MHCI is not associated with CD8 at time \( t \), and let \( P^*(t) \) denote the probability that TCR/pMHCI dissociation occurs after time \( t \) while the MHCI is associated with CD8 at time \( t \). Thus, \( P(t) + P^*(t) \) is the probability that TCR/pMHCI dissociation occurs later than \( t \); hence, \( k_{off} \) equals the rate of change of \(-\ln[P(t) + P^*(t)]\). The rate of change of \( P(t) + P^*(t) \) equals the following,

\[
\gamma_P - (\rho + k_{off}^s)P^s + P^s - (\gamma + k_{off}^a)P^a
\]  

(Eq. 6)

as a routine argument shows. Using this equation and the fact that MHCI/CD8 kinetics are much more rapid than the TCR/pMHCI kinetics (12), we obtain \( P^*P^s + P^* = \gamma_P \rho + \gamma \), which can be used to find the decay rate of \( P^* + P^s \) as shown here,

\[
k_{off} = k_{off}^s (\gamma + \rho) + k_{off}^a (\gamma + \rho)
\]  

(Eq. 7)

which we can rewrite as this,

\[
k_{off} = k_{off}^s \rho (k + K_{D,3D}) + k_{off}^a (k + K_{D,3D}) \rho
\]  

(Eq. 8)

by using Equation 5 and \( \kappa = |CD8| K_{D,3D}/K_{D,2D} \), where \( K_{D,3D} \) denotes the three-dimensional dissociation constant of the MHCI/CD8 interaction. Combining this equation with Equation 4 and \( k \gg K_{D,2D} \), an assumption warranted by our data, we obtain Equation 9,

\[
(k_{off}^s)^{1/3} \lambda = (3\mu)^{1/3} k_{off}^s + (3\mu)^{1/3} k_{off}^a K_{D,3D}/\kappa
\]  

(Eq. 9)

which is the linear relationship shown in Figs. 5B and 6B.

RESULTS

The Design of Mutations to Increase the Interaction between pMHCI and CD8—The D227K/T228A mutation in the \( \alpha2 \) domain of HLA A2 abrogates CD8 binding (8); the A245V mutation reduces CD8 binding by \( \geq 4 \)-fold (16). To develop similar HLA A2-based reagents with enhanced binding affinities for CD8\(\alpha\), we employed the multi-scale approach used by Glick et al. (42, 43). The algorithm was limited to four feature points. The surface of the CD8\(\alpha\) protein that forms contacts with HLA A2 (3) was systematically searched using amino acid side chains as “probes.” A Gln-115 to Glu mutation in the \( \alpha2 \) domain was selected for further molecular dynamics study. The wild type HLA A2 Q115E Oe1 atom forms a weak H-bond interaction with the CD8\(\alpha\) R4 N\(\gamma1\) atom. (The Oe1 . . . N\(\gamma1\) distance in the crystal structure is 3.18Å.) This interaction was replaced by a shorter H-bond between HLA A2 Q115E Oe1 and CD8\(\alpha\) R4 N\(\gamma1\) atoms; the molecular dynamics simulation showed that the Oe1 . . . N\(\gamma1\) average distance was 2.56Å and fluctuated from 2.42 to 2.82 Å. This short distance also indicates that the HLA A2 Q115E carboxylate and CD8\(\alpha\) R4 guanidinium moieties form a strong electrostatic interaction that is likely to increase the affinity of binding between the two biomolecules. SPR studies supported these predictions (Fig. 1). The human/murine hybrid HLA A2/H2-K\(\beta\) \(\alpha3\) domain fusion protein (A2/ K\(\beta\)), which has a substantially increased binding affinity for human CD8\(\alpha\), has been described previously (44).

Verification That Mutations Affect the pMHCI/CD8 Interaction without Altering the TCR/pMHCI Interaction—SPR was used to determine that the \( K_d \) values of the pMHCI/CD8 interaction for D227KT228A HLA A2, A245V HLA A2, wild type HLA A2, Q115E HLA A2, and A2/R\(\beta\) folded around the HTLV-1 epitope (LLFGYPVYV) were \( > 10,000, 498, 137, 98, \) and 10.9 \( \mu \)M respectively (Fig. 1A). The \( K_d \) values of the pMHCI/CD8 interaction for D227KT228A HLA A2, wild type HLA A2, Q115E HLA A2, and A2/R\(\beta\) folded around the HIV-1 epitope SLYNTVATL were shown to be \( > 10,000, 128, 87, \) and 9 \( \mu \)M, respectively (Fig. 1B). These substitutions in the \( \alpha3 \) or \( \alpha2 \) domain of the pMHCI molecule did not affect TCR binding (Fig. 1C). This spectrum of HLA A2 molecules that have normal TCR/pMHCI interactions but a range of pMHCI/CD8 interactions exceeding 1,000-fold was subsequently used to study the role of the pMHCI/CD8 interaction in the binding of pMHCI antigen.

The CD8 Interaction Determines the Pattern of pMHCI Tetramer Staining at Both Subnormal and Supranormal Affinities—Multimeric pMHCI molecules have recently revolutionized T cell immunology by enabling the direct visualization, enumeration, phenotyping, and sorting of T cells based on the antigen specificity of their TCRs (45–47). Emerging evidence suggests that the use of multimerized pMHCI antigens with decreased CD8 binding affinities enhances the specificity of these reagents in relation to background staining (48) and can be used to identify CTLs with a high sensitivity for antigen (49, 50). We used our panel of pMHCI tetramer complexes with differing affinities for CD8 to examine the staining of human CD8\(^+\) T cells specific for the HLA A2-restricted CMV pp65-derived epitope NLVPVMATV across a range of concentrations directly \( ex vivo \) (Fig. 2). These data show the utility of these reagents for staining direct \( ex vivo \) human PBMCs and eliminate the possibility that the mutations we have engineered into HLA A2 to alter CD8 binding inadvertently introduce new binding properties into the MHC class I molecule. A distinct antigen-specific population was identified with the corresponding pMHCI tetramers of Q115E HLA A2, wild type HLA A2, A245V HLA A2, and D227KT228A HLA A2; the magnitude of the detected population was equivalent for all of these reagents across all concentrations tested (Fig. 2). Similar results were obtained with PBMCs from two other donors with these CMV-specific reagents and in two further donors with pMHCI tetramers of wild type HLA A2, A245V HLA A2, and D227KT228A HLA A2; further experiments showed that the obtained population was equivalent for all of these reagents across all concentrations tested (Fig. 2).
as demonstrated by the mean fluorescence intensities of the CD8 tetramer+ subset. This trend extended to the extreme case in which all CD8+ T cells were stained by the A2/Kb tetrameric reagent (Fig. 2). Importantly, direct ex vivo staining of PBMC with CMV-specific tetramers showed that wild type and CD8 null reagents could identify a similar population of antigen-specific cells (Fig. 2). This finding suggests that these T cells bear a TCR with a relatively high affinity for cognate antigen (49).

The pMHCI/CD8 Interaction Affects pMHCI Tetramer Dissociation—The analysis of interaction kinetics between pMHCI and cell surface TCR has been hindered by the extremely short half-lives of such interactions (1–12 s at 25 °C). Tetrameric pMHCI molecules have considerably longer interaction times and allow a quantitative assessment of dissociation. We examined the decay of pMHCI tetramers with altered pMHCI/CD8 interactions but unaltered TCR/pMHCI interactions from the cell surface of an azide-poisoned CTL line that recognizes the HLA A2-restricted, HIV-1 p17 Gag-derived peptide SLYNTVATL. This epitope is frequently immunodominant in HLA A2+ HIV-infected patients, and the prevalent CTLs in line 868 have been shown previously to be expanded in the patient from whom the line was derived (32). The corresponding pMHCI proteins of A2/Kb, Q115E HLA A2, wild type HLA A2, A245V HLA A2, and D227K/T228A HLA A2 exhibit identical TCR binding but have different CD8 binding (8, 15, 16, 49) (Fig. 1B). All of these reagents stained a population of cells in the 868 CTL line (Fig. 3). The A2/Kb reagent stained all CD8+ T cells when used at high concentrations (Fig. 3). This result is consistent with our finding that this reagent stains all CD8+ human peripheral blood mononuclear cells regardless of their TCR specificity (49) (Fig. 2). When tetrameric A2/Kb is used at a low concentration, the cells with a TCR specific for the SLYNTVATL peptide appeared to out-compete other CD8+ cells for this reagent (Fig. 3; left column). Staining intensity with the D227K/T228A reagent was almost three times lower than with the wild type reagent (Fig. 3; right column). When stained with 0.5 μg/ml pMHCI was used (Fig. 3), consistent with the 2.5-fold difference in MPI we observed previously with this CTL line (8). Thus, these CTLs exhibit some dependence on the pMHCI/CD8 interaction for staining with pMHCI tetramers in contrast to the 003 CTL clone that recognizes the same antigen (8); these observations suggest that 003 CTL may bear a TCR with higher affinity for the cognate ligand and indicate that there is some variability between SLYNTVATL-specific CTLs in their requirements for the pMHCI/CD8 interaction to aid the binding of pMHCI tetramers.

The decay of pMHCI tetramers from the cell surface of 868
CTLs clearly correlates with the affinity of the pMHCI/CD8 interaction (Fig. 4A). To study this finding further, we examined CTL clone 003, which expresses a different SLYNTVATL-specific TCR than 868 CTL and is derived from a different patient (31, 32). This clone is believed to be the dominant clone that recognized this epitope in vivo (32). We have shown previously that wild type and D227K/T228A pMHCI tetramers exhibit a similar on-rate and stain these cells within 30 s (8). Using our panel of tetramers with altered CD8 binding properties, we confirmed that a decreased pMHCI/CD8 interaction leads to more rapid tetramer dissociation and that an increased pMHCI/CD8 interaction results in slower tetramer dissociation (Fig. 4B). Similar data were obtained with three further sets of CTLs (Fig. 4, C–E). Thus, the pMHCI/CD8 interaction affects the stability of the TCR/pMHCI interaction on the cell surface. The decay of pMHCI tetramers from 003 CTL (Fig. 4B) was slower than for 868 CTL (Fig. 4A). The former CTL might have a higher affinity TCR, consistent with our finding that 003 CTL are less dependent on CD8 for tetramer binding (8) than 868 CTL (Fig. 3).

The pMHCI/CD8 Interaction Stabilizes the TCR/pMHCI Interaction −2-Fold—The apparent off-rates in the tetramer dissociation experiments (Figs. 4–6) exhibit a wide variation; the D227K/T228A HLA A2 decay rate differs from that of wild type HLA A2 by a factor of −10. However, these differences in apparent off-rates do not translate directly into single-site off-rates, because tetramer kinetics involves TCR-bound forms of three different valencies (see Equations 1–3). In the regime where tetramer decay curves are virtually exponential, the true ratio of off-rates is the cube root of the ratio of apparent off-rates. In particular, the tetramer dissociation curve fit for D227K/T228A HLA A2 from 003 CTL indicates that \( \frac{(3/2)^{1/3}}{k_{off}} = 0.859 \text{ min}^{-1} \) (see Experimental Procedures for notation). The intercept of the straight line fit (Equation 9) shown in Fig. 5B yields \( (3/2)^{1/3}k_{off} = 0.398 \text{ min}^{-1} \). These data suggest that the maximum effect of the pMHCI/CD8 interaction is a prolongation by a factor of 2.16 of the mean TCR/pMHCI binding time. The fit also indicates that \( (3/2)^{1/3}k_{off}/k = 0.29 \text{ min}^{-1/3} \), whence \( k = 3 \text{ mm} \), which, in the interaction between a T cell and an antigen-presenting cell, would translate as \( 6 \times 10^4 \) free CD8 molecules in the contact area between a T cell and an antigen-presenting cell if we assume \( A = 30 \times 10^{-21} \text{ m}^2 \), where \( A \) is the area of the contact interface between the cells and \( \sigma \) is the “confinement length”; the free CD8 count equals \( A\sigma k \). Taken together, the tetramer decay experiments indicate that the pMHCI/CD8 interaction stabilizes by −2-fold the interaction between the TCR of HIV Gag-specific clone 003 and HLA A2/Kb-SLYNTVATL. The ac-
tual effect depends on the fraction of time spent by the MHC molecule in the CD8-bound form, and this fraction increases with increasing affinity. Thus, the wild type pMHCI/CD8 induces a stabilization factor of 1.95, which is close to the maximum of 2.16 by virtue of its low $K_D$ of 128 $\mu$M. A similar CD8-stabilization effect was observed for 868 CTLs (Fig. 6) and other CTLs with different functional sensitivities (summarized in Table I). We find that pMHCI tetramers rapidly induce the cell death of so-called “high avidity” CTLs (61). This may also be true when these reagents are used to sort-clone CTL. The failure of CTL clones with low functional avidity/sensitivity to stain with CD8 null tetramers (44) (Fig. 4) makes it impossible to calculate an apparent off-rate for these reagents from these clones. The lack of stability in the absence of a pMHCI/CD8 interaction precludes calculation of a CD8-mediated stabilization factor for such CTLs. Nevertheless, comparison of the off-rates of the other tetramers from the surface of CTL clone SLY-10 to those of other CTLs (Fig. 4) strongly suggests that the pMHCI/CD8 interaction provides a similar stabilization factor of ~2-fold for CTL clones of very low functional avidity.

**DISCUSSION**

The T cell surface glycoprotein CD8 interacts with nonpolymorphic regions of MHC, allowing a single pMHCI to bind both CD8 and TCR simultaneously (3). It has been reported that CD8 can increase the antigen sensitivity of CTL by a factor of $>10^6$ and significantly reduce the TCR triggering threshold required for CTL activation (8, 51). Several roles for CD8 in CTL activation have been proposed (reviewed in Refs. 52 and 53). CD8 could do the following: (i) stabilize TCR/pMHCI interactions at the CTL-target cell interface; (ii) play a major role in the topographical organization of cell surface TCR; and, (iii) act to recruit signaling molecules to the TCR-CD3 complex. Dissecting the role of CD8 in antigen recognition has traditionally been tackled using anti-CD8 antibodies. However, such antibodies can have both positive and negative effects on antigen recognition (27, 54) and are able to exert these functions in the absence of any interaction between pMHCI and CD8 (27).

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2 A. K. Sewell, unpublished data.
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Fig. 3. The effects of differing pMHCI/CD8 interactions on tetramer staining of 868 CTL. The CTL line 868 is derived from an HIV-1 infected donor and is specific for the HLA A2 restricted p17 Gag epitope SLYNTVATL. 2 × 10^6 868 cells per test were resuspended in 20 μl of PBS and stained with D227K/T228A HLA A2 (277/8KA), A245V HLA A2 (A245V), wild type HLA A2 (wildtype), Q115E HLA A2 (Q115E), or A2R/K a3 domain fusion (A2R/K). Tetramers folded around the SLYNTVATL peptide at either 0.5, 5 or 50 μg/ml for 20 min at 37 °C. Cells were then stained with CD8-allophycocyanin (clone SK1) for 30 min on ice, washed twice, and then resuspended in PBS. Data were acquired using a FACS Calibur flow cytometer and analyzed using CellQuest software. All HLA A2-SLYNTVATL tetramer-positive cells in the 868 CTL line bear a Vα12-2, Vβ5-6 TCR of identical sequence.

In addition, the use of antibodies directed against the CD8 molecule does not allow discrimination between the role of the pMHCI/CD8 interaction, direct coupling of CD8 to the TCR, or other possible roles of CD8 in antigen recognition. The role of CD8 has also been examined by transfecting CD8-negative T cell hybridomas with CD8α and CD8β (51, 55, 56). However, CD8 plays a pivotal role in the organization of cell surface TCRs (13, 27–29, 57–59) and the recruitment of essential signaling components to the cytoplasmic side of the TCR-CD3-ζ complex (6–9), thereby making impossible to study the role of the pMHCI/CD8 interaction in isolation with such systems. In view of these caveats, we developed the use of MHCI mutations that alter the affinity of CD8 binding without any effect on the TCR/pMHCI interaction in order to study the role of the pMHCI/CD8 interaction, because the precise impact of these mutations can be quantified by SPR (8). Many recent studies have used such molecules (8, 17, 27, 60, 61). These reagents have been used to show that the pMHCI/CD8 interaction enhances sensitivity to antigen by mediating complete phosphorylation of the TCR ζ chain (8). TCR engagement in the absence of a pMHCI/CD8 interaction results in preferential induction of only partially phosphorylated CD3-ζ (p21 phosphoform) and thus cannot effect rapid T cell activation (8). In the present study, we specifically address the role that CD8 plays in the stabilization and kinetics of the TCR/pMHCI interaction at the cell surface. This issue has been the source of much debate but is amenable to precise quantification with the point mutated recombinant proteins used herein.

The use of soluble pMHCI proteins enables TCR/pMHCI interactions and kinetics at the cell surface to be studied without interference from other adhesion or co-stimulatory molecules. In the monomeric form the use of this method is complicated by the extremely short interaction half-life; however, increasing the valency of these reagents by avidin-biotin-based tetramerization significantly increases the cumulative avidity and produces reagents that are valuable tools for the identification and phenotyping of antigen-specific CTLs (45, 46). The effect of the pMHCI/CD8 interaction on the ability of pMHCI tetrameric reagents to form stable interactions with cell surface TCRs has been extensively investigated. Several studies have demonstrated that anti-CD8 antibodies act to block pMHCI tetramer binding to both human and murine CTLs (62–64). In these studies, it was assumed that anti-CD8 antibodies exert their effects by blocking the pMHCI/CD8 interaction, leading to the conclusion that this interaction is “critical” for the stable binding of pMHCI tetrameric reagents to cell surface TCRs. However, we have since shown that anti-CD8 antibodies can reduce tetramer binding even in the absence of a pMHCI/CD8 interaction (27). Anti-CD8 antibodies need not therefore block tetramer binding by interfering with the pMHCI/CD8 interaction and thus cannot be used to define the effect of this interaction on the binding avidity of pMHCI tetramers. We have recently reinforced these findings by showing that some anti-CD4 antibody clones inhibit the binding of pMHCII tetramers in several systems.3

Introducing the a3 domain mutation D227K/T228A into HLA A2 has been shown to abrogate the pMHCI/CD8 interaction (K_D of >10,000 μM) without affecting the TCR/pMHCI interaction (8). We have shown that “CD8 null” tetramers bearing this mutation can stain human anti-viral CTL clones efficiently and at an intensity and on-rate similar to that of staining with wild type tetramers (8). For many human anti-viral CTLs, the TCR/pMHCI interaction is almost 100 times stronger than the pMHCI/CD8 interaction (16) and of significantly longer duration; therefore, it is not surprising that the requirement for the latter interaction in the stable cell surface binding of pMHCI tetramers is minimal. In this study, we confirm that CD8 null tetramers can efficiently stain human anti-viral CTL in vitro. Furthermore, we show that both wild type and CD8 null pMHCI tetramers efficiently stain similar populations of anti-CMV and anti-EBV CTLs directly ex vivo (Fig. 2, and data not shown). Thus, in accordance with our in vitro studies with human and murine CTL (8, 27, 49), we find that the pMHCI/CD8 interaction is not uniformly essential for multimer binding to cell surface TCRs. Previous studies have shown that the introduction of a3 domain mutations that reduce the pMHCI/CD8 interaction can significantly decrease the level of pMHCI tetramer binding in human and murine systems and the level of murine pMHCI

3 L. Wooldridge and T. J. Scriba, unpublished data.
binding as assessed by photoaffinity labeling (14, 17, 58, 65). Our data demonstrating that the pMHCI/CD8 interaction need not be critical for stable pMHCI multimer binding may at first seem at variance with these studies. However, it is now clear that CTLs exhibit a range of dependence on the pMHCI/CD8 interaction for the stable cell surface binding of pMHCI tetramer.
rameric complexes. We have shown previously that D227K/T228A CD8 null tetramers selectively stain only those CTLs with a high sensitivity for antigen (49). Wild type reagents can stain low avidity CTL efficiently; however, CD8 null reagents stain low avidity anti-viral and tumor-specific CTLs poorly or not at all (49). Therefore, the dependence on the pMHCI/CD8 interaction for stable tetramer binding correlates with the functional avidity of the CTL and is thought to reflect the intrinsic affinity of the TCR for the pMHCI ligand, along with other factors such as cell surface organization and density of the TCR (49, 50, 66). In contrast, the pMHCII interaction with the CD4 coreceptor is significantly weaker than the pMHCI/CD8 interaction (67, 68). Early studies in which CD4 and a mutant CD4 without a capacity for cytoplasmic signaling were expressed in T cell hybridomas lacking endogenous CD4 concluded that CD4 has a very minor role as an adhesion molecule in T cell activation (69). This finding has been upheld by more recent reports showing that CD4 does not aid the stabilization of the TCR/pMHCI interaction at the cell surface (70–72).

Our data point to a reconciliation of the apparently disparate findings regarding the requirement for the pMHCI/CD8 interaction in the stable binding of pMHCI tetramers. In attempting such a reconciliation, it is important to treat results gained using anti-CD8 antibodies with caution because these reagents appear to have multiple effects, some of which are independent of the interaction between pMHCI and CD8 (27). It is also important to take the increased affinity of the pMHCI/CD8 interaction in mouse as compared with that in human (8, 73) into account. However, even when such factors are taken into consideration, there appears to be variability within the human (27, 49) and murine (44, 49) systems with regard to the requirement for CD8-mediated stabilization for pMHCI multimer binding. The TCR/pMHCI interaction is of short duration (<1–12 s at 25 °C). Tetramers derive their high avidity from the large probability that a monovalently bound tetramer will bind bivalently before the single bound site dissociates; this probability is large if the association rate for further sites is much greater than the single-site dissociation rate. This is certainly the case for the tetramers used in this study, as evidenced by the exponential tetramer dissociation curves. Thus, tetramerization of the TCR/pMHCI interaction increases the bound half-life by hundreds of fold (74), as all pMHCI molecules need to be unligated simultaneously for the tetramer to dissociate from the cell surface. As described above, stable cell surface adhesion of pMHCI tetramers has an empirical requirement for the monomeric interaction to be of sufficient duration to allow a further monomer in the complex to interact with another TCR prior to release of the original interaction. Presumably, strong TCR/pMHCI interactions, such as those of immunodominant human anti-viral CTLs, exceed this minimal requirement per se. We have shown that TCR and CD8 cooperate in binding pMHCI at the cell surface (Fig. 4). The pMHCI/CD8 interaction delays the dissociation of the TCR/pMHCI interaction by a factor of 2 (Figs. 5 and 6 and Table I) and can thus enable weaker TCR/pMHCI engagements to attain the minimal half-life for stable binding of tetrameric reagents to the CTL surface.
The kinetic proofreading model of T cell activation (20, 21) in which the $k_{off}$ of the TCR/pMHCI interaction and, hence, its half-life, appears to be the principal feature determining the biological outcome of TCR ligation is widely accepted. Recent rigorous testing of this model reinforces its relevance but reverses the model to account for how low level undetectable signaling induced by weak TCR ligands can trickle down to affect T cell activation (75) and also to account for how a few ligands can be somewhat more stimulatory than predicted on the basis of the half-life of the TCR/pMHCI interaction alone by the inclusion of a value for heat capacity (76). The potential role of CD8 in stabilizing this interaction is therefore of considerable biological importance and has been the subject of intense debate (11–14). We have shown previously that pMHCI multimers are rapidly internalized by CTL that bear a cognate TCR (77). This process can be prevented by staining on ice in the presence of azide as described under “Experimental Procedures.” If tetrameric pMHCI is prevented from internalizing and re-binding using an anti-MHCI blocking antibody or “cold” unlabeled tetramer, then it is possible to examine tetramer decay from the cell surface (78). Using human anti-viral CTL and pMHCI tetramers with abrogated, reduced, normal, slightly enhanced, and greatly enhanced CD8 binding affinities but unaltered TCR binding, we find that the pMHCI/CD8 interaction can significantly affect the dissociation rate of pMHCI tetramers. Abrogating the pMHCI/CD8 interaction significantly lowers the half-life of pMHCI tetramer binding to cell surface TCR (Fig. 4). Increasing the pMHCI/CD8 interaction without altering the TCR/pMHCI interaction (Fig. 1) results in a pMHCI tetramer that binds to the T cell surface with greater stability than the wild type molecule (Fig. 4). We modeled the contribution of the pMHCI/CD8 interaction to stabilization of the TCR/pMHCI interaction and found that the pMHCI/CD8 interaction stabilizes the monomeric interaction between the TCR of HIV-1 Gag-specific 003 and 868 CTLs and HLA A2-SLYNTVATL by ∼2-fold. A similar value was also determined from the decay from other CTL (summarized in Table 1). This factor did not appear to vary with the functional avidity of the CTL or the epitope recognized. It may be shown that the calculated stabilization factor is not dependent on the assumption $CTL$ or the epitope recognized. It may be shown that the calculated stabilization factor did not appear to vary with the functional avidity of the CTL or the epitope recognized. Therefore, we conclude that the CD8 stabilization effect can alter the order of that ranking, allowing CTLs to focus their functional avidity on a ligand by adjusting CD8 expression levels.

In summary, we have used a range of pMHCIIs with altered CD8 binding but unaltered TCR binding to examine the TCR/ pMHCI/CD8 interaction at the cell surface. These experiments allow an assessment of cooperative binding not possible in previous biophysical and structural studies using soluble molecules (3, 12, 13). We show that the TCR and CD8 bind to pMHCI cooperatively at the cell surface. Modeling for the monomeric TCR/pMHCI/CD8 interaction indicates that CD8 provides a stabilization factor of ∼2 that is applicable across all systems tested. The requirement for CD8 to stabilize the TCR/ pMHCI interaction beyond a threshold sufficient for TCR triggering or stable binding of multimeric pMHCI to cell surface TCR is minimal with strong TCR ligands (15) but becomes increasingly apparent as the TCR/pMHCI half-life decreases (49, 50), consistent with model predictions (79). The 2-fold stabilization effect provided by the pMHCI/CD8 interaction is expected to enhance T cell activation per se. The TCR triggering rate has been found to depend non-monotonically on the off-rate, with an optimum positioned at a point where $1/k_{off}$ corresponds to the TCR triggering threshold (79, 80). Reducing $k_{off}$ by a factor of 2 may in fact have a negative impact on ligands with off-rates too slow for optimal stimulation. However, the vast majority of ligands will have off-rates too fast for optimal stimulation of a given TCR, and such ligands will increase in TCR triggering efficacy when $k_{off}$ is reduced by half. Indeed, weak agonist variant peptides cannot be recognized in the absence of CD8/CD8 interaction (Fig. S3 in the supplemental data found in the on-line version of this article). Overall, these findings suggest that CD8-mediated stabilization of the TCR/pMHCI interaction contributes to cell cross-reactivity and promiscuity (81), an effect that might be amenable to therapeutic intervention.

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REFERENCES

1. Davis, M. M., Boniface, J. J., Reich, Z., Lyons, D., Hampel, J., Arden, B., and Chien, Y. (1998) Annu. Rev. Immunol. 16, 525–544
2. Rudolph, M. G., and Wilson, I. A. (2002) Curr. Opin. Immunol. 14, 52–65
3. Gao, G. F., Torchia, J., Gerth, U. C., Wyer, J. R., McMichael, A. J., Staat, D. I., Bell, J. L., Jones, E. Y., and Jakobsen, B. K. (1997) Nature 387, 630–634
4. Kern, P. S., Teng, M. K., Smolyar, A., Liu, J. H., Liu, J., Hussey, R. E., Spoerl, R., Chang, H. C., Reinherz, E. L., and Wang, J. H. (1998) Immunity 9, 519–530
5. Janeway, C. A., Jr. (1992) Annu. Rev. Immunol. 10, 645–674
6. Veillette, A., Bookman, M. A., Horak, E. M., and Bolen, J. B. (1988) Cell 55, 301–308
7. Chalupny, N. J., Ledbetter, J. A., and Kathavas, P. (1991) EMBO J. 10,
