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The CD8 T Cell Coreceptor Exhibits Disproportionate Biological Activity at Extremely Low Binding Affinities*

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T lymphocytes recognize peptides presented in the context of major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells. Recognition specificity is determined by the $\alpha\beta$ T cell receptor (TCR). The T lymphocyte surface glycoproteins CD8 and CD4 enhance T cell antigen recognition by binding to MHC class I and class II molecules, respectively. Biophysical measurements have determined that equilibrium binding of the TCR with natural agonist peptide-MHC (pMHC) complexes occurs with K_D values of 1–50 μM . The pMHCI/CD8 and pMHCI/CD4 interactions are significantly weaker than this ($K_D > 100 \mu\text{M}$), and the relative roles of TCR/pMHC and pMHC/coreceptor affinity in T cell activation remain controversial. Here, we engineer mutations in the MHCI heavy chain and β_2 -microglobulin that further reduce or abolish the pMHCI/CD8 interaction to probe the significance of pMHC/coreceptor affinity in T cell activation. We demonstrate that the pMHCI/CD8 coreceptor interaction retains the vast majority of its biological activity at affinities that are reduced by over 15-fold ($K_D > 2 \text{ mM}$). In contrast to previous reports, we observe that the weak interaction between HLA A68 and CD8, which falls within this spectrum of reduced affinities, retains substantial functional activity. These findings are discussed in the context of current concepts of coreceptor dependence and the mechanism by which TCR coreceptors facilitate T cell activation.

T lymphocytes recognize protein antigens in the form of short peptides presented in association with major histocompatibility complex (MHC)¹ molecules on the surface of target cells. Antigen specificity is conferred by the T cell receptor (TCR), whose highly variable complementarity determining regions interact with the peptide-binding platform of the MHC molecule (1, 2).

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¹ The abbreviations used are: MHC, major histocompatibility complex; TCR, T cell receptor; pMHC, peptide-major histocompatibility complex; $\beta_2\text{m}$, β_2 -microglobulin; RANTES, regulated on activation normal T cell expressed and secreted; CTL, cytotoxic T lymphocytes; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; IFN, interferon; MIP-1 β , macrophage inflammatory protein-1 β .

The peptide-MHC complex (pMHC) also interacts with the T cell surface glycoproteins CD8 and CD4, which bind to invariable regions of the MHC class I and II molecules, respectively (3–7). The binding sites for CD8 and CD4 are separate from the TCR-recognized (2), peptide-binding domains of MHC molecules, and allow a single MHC molecule to be bound simultaneously by both TCR and either CD8 or CD4 (5, 6). The cytoplasmic domains of CD8 and CD4 are known to interact with the T cell-specific intracellular protein-tyrosine kinase p56^{lck} (8, 9). This kinase is critical for the initiation of the TCR-mediated signal transduction cascade (10). The recruitment of essential signaling components to the cytoplasmic side of the TCR by simultaneous binding of either CD8 or CD4 to the same pMHC enhances TCR-mediated signal transduction (11–13). Thus, CD8 and CD4 act as coreceptors for antigen in concert with the antigen-specific TCR during the process of T cell recognition and activation.

Whereas CD8 and CD4 appear to have similar biological roles, they have little structural similarity (14, 15). CD8 is present on the lymphocyte cell surface as either a disulfide-bonded $\alpha\alpha$ homodimeric or $\alpha\beta$ heterodimeric molecule. CD8 $\alpha\beta$ predominates on cells expressing pMHCI-specific $\alpha\beta$ TCRs (16). The CD8 α chain recruits signaling components to the cytoplasmic side of the TCR/pMHC interaction (17, 18) and provides the majority of the binding energy in the CD8 $\alpha\beta$ heterodimer (19). The globular head domains of murine CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ bind to pMHCI with similar affinity (20). Cell-cell adhesion assays have shown that human CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ mediate adherence to pMHCI equally (21). Despite similar affinities for pMHCI, $\alpha\beta$ heterodimer is more efficient than $\alpha\alpha$ homodimer in promoting the response to antigen (22). This suggests a distinct function for CD8 β . Recent evidence throws light on this role. First, it has been shown that CD8 β is palmitoylated at a membrane-proximal cysteine (23). This palmitoylation mediates the partitioning of CD8 in ordered, cholesterol- and sphingolipid-enriched, membrane microdomains known as lipid rafts or detergent-insoluble membranes (23). The short CD8 β cytoplasmic domain also enables constitutive association with the TCR/CD3 complex and recruits this antigen recognition complex to lipid rafts (24). Rafts exclude inhibitory phosphatases, such as CD45, and have been shown to be privileged sites for the induction of the TCR-associated signal transduction cascade (25–29). CD8 β -mediated recruitment of the TCR to lipid rafts might therefore explain the greater efficiency of CD8 $\alpha\beta$ in promoting the response to antigen. Second, it has been shown that the pMHCI/CD8 interaction is not fixed but can be developmentally regulated by glycosylation (30). Glycans on the CD8 β stalk appear to modulate the ability of the

distal binding surface of the dimeric CD8 globular head domains to bind pMHCI (31). This differential glycosylation, leading to altered pMHCI binding, highlights a further potential role of CD8 β . In contrast to CD8, CD4 is a single polypeptide. The cytoplasmic domain of CD4, like that of CD8 α , is known to interact with p56^{lck} (9). Further work is required to determine whether the single polypeptide of CD4 also incorporates the roles recently ascribed to the CD8 β chain.

The TCR and CD8 or CD4 coreceptors bind peptide-MHC independently and with distinct kinetics (32, 33). The affinity of pMHC/coreceptor interactions are at the low extreme of those described for cell-cell interaction molecules (7, 32). Equilibrium binding of human CD8 $\alpha\alpha$ has been measured with several different HLA A, B, and C gene products and occurs at K_D values of greater than 100 μ M (34). The pMHCI/CD4 interaction is similarly weak ($K_D = 200 \mu$ M) (33). Here we examine the role of CD8 in antigen recognition using mutational changes in the $\alpha 3$ domain of MHCI and the β_2 -microglobulin subunit. These studies have enabled us to assess the contribution of the binding energy provided by the extracellular pMHCI/CD8 interaction to the activation of human anti-viral cytotoxic T lymphocytes (CTL).

EXPERIMENTAL PROCEDURES

Inclusion Body Preparation—Biotin-tagged HLA-A2 and HLA-A68² heavy chains were expressed under the control of a T7 promoter as insoluble inclusion bodies in *Escherichia coli* strain BL21(DE3)pLysS (Novagen). Isopropyl-1-thio- β -D-galactopyranoside-induced *E. coli* were lysed by repeated freeze/thaw cycles to release inclusion bodies that were subsequently purified by washing with a 0.5% Triton X-100 buffer (Sigma) as described previously (35). The D227K/T228A mutation in the $\alpha 3$ domain of HLA A2 has been shown to abrogate CD8 binding (12); the corresponding A245V mutation reduces CD8 binding 5-fold (34). D227K/T228A and residue 245 mutants of HLA A2 and HLA A68 heavy chains, together with β_2 -microglobulin (β_2 m) and K58E β_2 m (36), were produced using the same expression system.

Production of Soluble Peptide-MHC Class I—Soluble biotinylated MHC class I monomers were produced as described previously (36). Briefly, either HLA A2 or HLA A68 heavy chain and β_2 m inclusion body preparations were denatured separately in 8 M urea buffer (Sigma) and mixed at a 1:1 molar ratio. pMHCI was refolded in 2-mercaptoethylamine/cystamine (Sigma) redox buffer with added synthetic peptide (Research Genetics Invitrogen Corp., Huntsville, AL). HLA A2 heavy chains were refolded with the HIV p17 Gag epitope SLYNTVATL (residues 77–85) or the influenza matrix protein GILGFVFTL (residues 58–66). HLA A68 heavy chain was refolded with the HIV-1 Tat epitope ITKGLGISYGR (37). Following buffer exchange into 10 mM Tris, pH 8.1, refolded monomer was purified by anion exchange. Purified monomers were biotinylated as previously described (35) using *d*-biotin (Sigma) and BirA enzyme. Excess biotin was removed by gel filtration.

Surface Plasmon Resonance—SPR analysis of biomolecular interactions between the MHC class I monomers and human soluble CD8 $\alpha\alpha$ (sCD8 $\alpha\alpha$) was performed on a BIAcore 3000TM (BIAcore AB, St. Albans, UK). sCD8 $\alpha\alpha$ was prepared as described previously (36, 38). For analysis, all proteins 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. Standard amine coupling kit (BIAcore AB) was used to activate the surface of a research grade CM5 sensor chip (BIAcore AB). Streptavidin was covalently coupled to the chip surface via primary amines by injecting a 0.2 mg/ml streptavidin solution (Sigma) diluted in 10 mM sodium acetate, pH 4.5, over the surface. Biotinylated monomers were immobilized onto the chip surface at ~1000 response units (RU) in each flow cell. Serial dilutions of sCD8 $\alpha\alpha$ diluted in HBS-EP buffer were flowed over the chip to generate equilibrium binding data. Data were analyzed using BIAeval, Microsoft Excel, and Origin version 6.1 (Microcal software). K_D values were calculated by non-linear curve fitting assuming 1:1 Langmuir binding ($A + B \leftrightarrow AB$) using the equation $AB = B \times AB_{max} / (K_D + B)$ and were verified by linear regression analysis of Scatchard plots.

Tetramerization and Flow Cytometry—Biotinylated pMHCI mono-

mers were conjugated by addition of extravidin-R-phycoerythrin (Sigma) at a pMHCI:extravidin molar ratio of 4:1 to produce tetrameric pMHCI complexes. Once prepared, tetramers were stored in the dark at 4 °C. 1×10^5 CD8⁺ CTL in 20 μ l of phosphate-buffered saline (PBS) were stained with 1 μ g of phycoerythrin-tetramer (pMHCI content) for 20 min at 37 °C, washed twice in PBS, and then analyzed using a FACScalibur flow cytometer (BD Biosciences) with Cell Quest software. All pMHCI multimers used in this study were made fresh for the week of use from pMHCI monomers stored at -80 °C to avoid the effect of the stability differences documented in Fig. 3. Where comparisons were made between the activation induced by different pMHCI multimers they were tested by FACS to ensure that they exhibited an equal ability to bind to cell surface TCR.

ELISA for Soluble Lymphokines—Immortalized B cell lines were washed once in RPMI media and pulsed with peptide for 1 h at 37 °C, 5% CO₂. Peptide-pulsed B cell lines and CTL were each washed twice in RPMI and brought to a concentration of 5×10^5 cells/ml in RPMI medium supplemented with 2% fetal calf serum. 5×10^4 CTL and 5×10^4 B cells were incubated together in a 200 μ l final volume for 4 h or 30 min at 37 °C, 5% CO₂ in 96-well U-bottomed plates. Supernatant was harvested with care not to disturb cells and assayed for MIP-1 β and RANTES by ELISA (R & D Systems). Standard deviation from the mean of two duplicate assays is shown.

ELISpot Assay for Single-cell IFN- γ Release—96-Well polyvinylidene difluoride-backed plates (Millipore) were coated with IFN- γ capture antibody 1-DIK (Mabtech) at 15 μ g/ml, and blocked with RPMI containing 10% fetal calf serum. 5×10^4 B cells and 2×10^2 CTL \pm peptide were applied to duplicate wells of pre-coated plates and incubated for 3 h at 37 °C, 5% CO₂. Plates were washed 6 times with PBS (Sigma) at room temperature and incubated for 90 min with 1 μ g/ml anti-IFN- γ mAb-7B6-1 biotin (Mabtech). After 6 further washes, a 1:1000 dilution of alkaline phosphatase-conjugated streptavidin (Mabtech) was added and incubated at room temperature for 40 min. After further washing, chromogenic AP substrate (Bio-Rad) was added for 30 min and development was stopped by rinsing twice with water. Spots were counted mechanically using an ELISpot Reader system ELR02 (Autoimmun Diagnostika; Strassberg). Standard deviation from the mean of two duplicate assays is shown.

Stimulation of CTL for Subsequent Immunoblotting—CTL were washed twice in RPMI and incubated overnight in RPMI with 10% fetal calf serum. The following day, fetal calf serum was washed off with two changes of RPMI and 10^6 CTL were resuspended in 10 μ l of RPMI. After 10 min at 37 °C in 5% CO₂, CTL were stimulated by incubation with 1 μ g/ml tetramer for 3 min. The reaction was stopped by washing once with 0.5 ml of ice-cold PBS, and re-suspending the pellet in cold lysis buffer (140 mM NaCl, 20 mM Tris, pH 8.0, 10 mM sodium fluoride, 2 mM EDTA, 20% glycerol, 1% IGEPAL, 1 mM Na₃VO₄, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin) at 5×10^7 cells/ml.

Antiphosphotyrosine Immunoblots—Cells were lysed on ice for 30 min, then the nuclear fraction was pelleted by centrifugation at $16,000 \times g$ for 15 min. The remaining lysate was aspirated and added to an equal volume of SDS loading buffer (350 mM Tris, pH 6.8, 350 mM SDS, 30% glycerol, 600 mM dithiothreitol, 175 μ M bromophenol blue). The sample was boiled for 6 min with agitation and then loaded into a 12% SDS-PAGE protein gel for electrophoresis at 100 V for 16 h. The gel, filter papers (Bio-Rad), and nitrocellulose (Amersham Biosciences) of matching size were equilibrated in ice-cold transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol) for 10 min. Protein was transferred from the gel by electrophoresis at 25 V for 50 min. The gel was stained with Coomassie Blue and the membrane stained with Ponceau S to verify transfer and equal protein loading. Ponceau S was washed off with distilled water and then wash buffer (PBS, 0.05% Tween). The membrane was blocked for 1 h with wash buffer containing 1% bovine serum albumin, washed, and incubated for 4 h with mouse anti-phosphotyrosine antibody clone 4G10 (Upstate Biotechnology, 1:1000 in wash buffer, 0.1% bovine serum albumin). The membrane was washed again with 3 changes of wash buffer for 10 min each, and incubated with sheep anti-mouse peroxidase-linked secondary antibody (Amersham Biosciences, 1:2000 in wash buffer, 2.5% milk powder) for 1.5 h. After 3 further washes the blot was developed using chemiluminescent substrate Supersignal Pico (Perbio). All washes and incubations with antibody were performed at 4 °C. At least 48 h after development, blots were re-probed for total cellular ZAP 70 to control for protein loading. To re-probe, the membrane was re-hydrated in wash buffer then incubated with rabbit anti-human ZAP 70 primary antibody (Autogen Bioclear, 1:1000 in wash buffer, 2.5% milk powder) for 4 h. The blot was washed 3 times and incubated with peroxidase-linked anti-rabbit secondary antibody (Autogen Bioclear, 1:1000 in wash buffer, 2.5% milk

² HLA A2 and HLA A68 are used to define HLA A*0201 and HLA*6801 throughout this article.

powder) for 1.5 h, then washed again and developed as above.

Manufacture of C1R-HLA A68-expressing Cells—Human HLA A68 cDNA was obtained by reverse transcription of total RNA extracted from human peripheral blood mononuclear cells and PCR amplification of the specific cDNA. The cDNA was cloned as a *Bam*HI-*Eco*RI fragment in the retroviral vector pLNSX (Clontech) to obtain the vector A6801-pLNSX. The D227K/T228A mutations were introduced by site-directed mutagenesis (Stratagene). The plasmids were introduced by calcium phosphate precipitation into the xenotropic packaging cell line PG13 (American Type Culture Collection) for the production of recombinant retroviral vectors. After 2 weeks of selection in G418 (800 μ g/ml; Sigma) supernatant from producing cells was harvested and used for transduction of C1R (39) target cells. 5×10^6 cells were transduced using 5 ml of viral supernatant in the presence of protamine at a concentration of 8 μ g/ml (Sigma) for 16 h and subsequently grown in selective medium containing G418 (400 μ g/ml) for 2 weeks. Cell surface expression of HLA A68 and D227K/T228A HLA A68 was quantified by flow cytometry. 10^6 retrovirally transduced cells were washed in PBS, 0.1% bovine serum albumin and incubated with 1 μ g of anti-HLA class I (W6/32) antibody for 30 min on ice. The cells were washed twice in PBS, 0.1% bovine serum albumin and incubated with a fluorescein-conjugated secondary antibody for 30 min on ice. Cells were washed twice and analyzed by flow cytometry as described above. Correct refolding of HLA A68 and D227K/T228A HLA A68 on the surface of C1R cells was confirmed by CTL assay. Both of these cells were able to present antigen to HLA A68-restricted CTL when pulsed with very high levels of peptide antigen (>10 mM) and used in ELISpot assays as described above.

RESULTS

Generation and Validation of pMHCI and β_2 m Mutations That Reduce the CD8 Coreceptor Interaction—The CD8 coreceptor makes contacts with both the $\alpha 3$ domain, MHC class I heavy chain and β_2 m (5). We have described mutations in the MHC heavy chain/ β_2 m complex that reduce the affinity of the pMHCI/CD8 interaction without affecting the integrity of the TCR-binding platform (12, 34, 36). The binding of CD8 to D227K/T228A HLA A2 is below the current limits of detection by SPR (12). We simulated non-linear curves on our data by using the equation $y = (P1 \times x)/(P2 + x)$ and are confident we would be able to detect binding of CD8 to pMHCI with a K_D as low as 10 mM. Consequently we are confident that the D227K/T228A mutation reduces the binding of CD8 to HLA-A2 by over 50-fold. We have also previously determined that wild type and D227K/T228A HLA A2 bind to an HLA A2 influenza matrix-specific TCR (JM22) and HLA A2 HTLV-1 Tax-specific TCR (A6) equally well (12). Similarly we have confirmed that the K58E substitution in β_2 m has no effect on the binding of TCR (36). The interaction of soluble CD8 α (sCD8) with HLA A2 complexed with the HIV-1, p17 Gag-derived peptide SLYNTVATL, and β_2 m containing the K58E substitution was reduced by more than 15-fold compared with the SLYNTVATL-HLA A2 complex folded with wild type β_2 m ($K_D > 2$ mM) (Fig. 1). We have reported that HLA A2 complexed with the influenza matrix-derived peptide GILGFVFTL and K58E β_2 m exhibits greatly reduced binding to sCD8 (36). This result was confirmed here to ensure comparability of results (Fig. 1). Curiously, wild type HLA A2 complexed with wild type β_2 m and GILGFVFTL peptide bound sCD8 with a lower affinity than HLA A2 presenting other peptides (data not shown). This result is in agreement with previous measurements that show that ILKEPVHGV-HLA A2 (34) has a higher affinity for CD8 ($K_D = 130$ μ M) than HLA A2 folded with GILGFVFTL peptide ($K_D = 195$ μ M) (36). It is not clear whether this effect is due to peptide-induced structural changes transmitted to the HLA A2 $\alpha 3$ domain or an artifact of protein folding with the GILGFVFTL peptide. As with the wild type complexes, GILGFVFTL-HLA A2/K58E β_2 m exhibited slightly lower CD8 binding than SLYNTVATL-HLA A2/K58E β_2 m (Fig. 1).

Mutations in pMHCI and β_2 m That Reduce the CD8 Coreceptor Interaction Do Not Reduce the Binding of Peptide—MHC

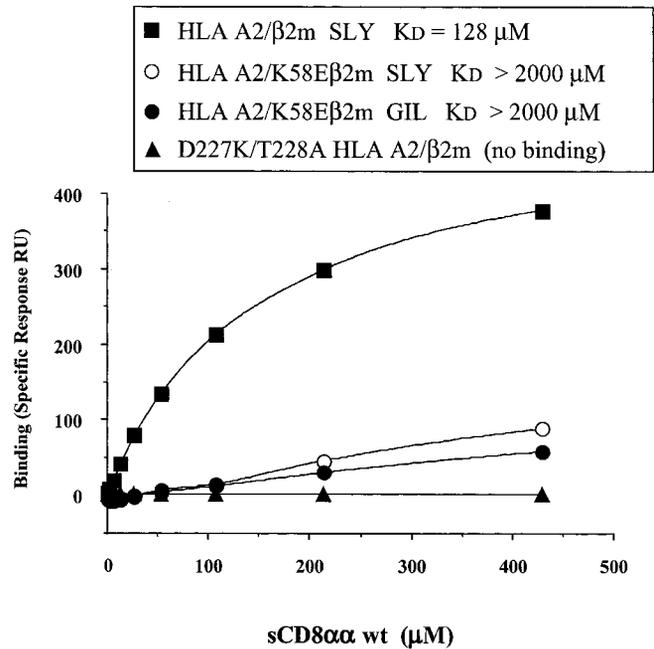


FIG. 1. The effect of K58E β_2 m mutation on the affinity of sCD8 α binding to HLA A2 monomers. SLYNTVATL-HLA A2/ β_2 m (positive control), SLYNTVATL-HLA A2/K58E β_2 m, GILGFVFTL-HLA A2/K58E β_2 m, and SLYNTVATL-D227K/T228A HLA A2/ β_2 m (negative control) biotinylated monomeric complexes were loaded onto separate flow cells of a streptavidin-coated research grade CM5 chip (BIAcore, AB) at ~ 1000 response units (RU). sCD8 α was injected at increasing concentrations through all 4 flow cells. For each concentration of sCD8 α (μ M), the specific binding was calculated as the difference between the responses at equilibrium in the experimental flow cells compared with the negative control. K_D of the interaction between sCD8 α and SLYNTVATL-HLA A2/ β_2 m (positive control) was calculated by plotting specific binding against the sCD8 α concentration for non-linear curve analysis. Linear Scatchard plot analysis was also performed (data not shown). SLYNTVATL-HLA A2/K58E β_2 m and GILGFVFTL-HLA A2/K58E β_2 m mutants showed very weak interaction with sCD8 α relative to that seen with SLYNTVATL-HLA A2/ β_2 m (positive control). Even at high sCD8 α concentration specific binding with the K58E mutants does not approach equilibrium. Using the equation $y = (P1 \times x)/(P2 + x)$ simulated non-linear curves were plotted to represent K_D values ranging between 2 and 5 mM. Specific binding measured for both K58E mutants was then plotted against sCD8 α concentration and a comparison with the simulated curves was made. Using this approach it was suggested that the K_D of the interaction between sCD8 α and both K58E mutants is >2 mM but <4 mM.

class I structures show that the $\alpha 3$ domain mutations utilized in this study are removed from the $\alpha 1/\alpha 2$ peptide-binding platform of these molecules and therefore are unlikely to have direct effects on the binding of peptide (5). The *in vitro* refolding efficiency of pMHCI is highly dependent on the ability of peptide to bind (40). We have taken advantage of this and used such assays as a measure of peptide binding (40). We find that this technique is more sensitive than traditional ways of measuring peptide binding (41, 42). The efficiency of *in vitro* refolds with D227K/T228A and A245V HLA A2 is equal to that of the wild type molecule (data not shown). The efficiency of *in vitro* refolding with K58E β_2 m is actually slightly better than that with the wild type molecule. This increased efficiency is likely due to the increased β_2 m/MHCI heavy chain interactions that we have described previously (36). We observe similar refolding efficiency of wild type HLA A68 and the mutated HLA A68 molecules used in this study (data not shown). In addition, we have made mammalian expression constructs for green fluorescent protein-HLA A2 fusion proteins. Some HLA A2 mutations are observed to affect transport of such proteins to the cell surface, possibly as a result of reduced peptide binding. D227K/T228A and A245V HLA A2 are observed to traffic to the cell

surface normally by FACS and confocal microscopy (data not shown). We have also previously determined that the C1R-HLA A2 cells used in this study express identical levels of cell surface HLA A2 by FACS analysis with HLA A2 conformation-specific antibody (12). Differences in peptide loading of mutant MHC1 should demonstrate themselves by a difference in cell surface expression. As such differences in surface expression are not observed, and all molecules fold as well as the wild type molecule *in vitro*, it seems highly likely that our mutant MHC1 molecules bind peptide as well as the wild type molecules.

The pMHC1/CD8 Coreceptor Interaction Can Be Substantially Reduced yet Remains Biologically Significant—We have shown previously that the A245V substitution in the $\alpha 3$ domain of HLA A2 reduces the pMHC1/CD8 interaction by 5-fold (34). We tested whether this reduced binding maintained biological activity. The earliest biochemical changes known to occur upon TCR engagement of pMHC antigen involve the activation of protein-tyrosine kinases associated with the cytoplasmic domains of the TCR/CD3/coreceptor complex (43). These signaling events can be detected within seconds of antigen engagement and peak within the first 10 min of activation before rapidly declining (44). We have shown that the human CD8 coreceptor effects CTL activation and antigen sensitivity primarily by mediating these phosphorylation events including the complete phosphorylation of the TCR ζ chain (12). The B cell line C1R (39) expressing full-length HLA A2 was capable of inducing full phosphorylation of the TCR ζ chain when presenting cognate peptide as expected; similar levels of ζ phosphorylation were observed with C1R cells expressing A245V substituted HLA A2 (Fig. 2A). In contrast, HLA A2 with a D227K/T228A double substitution, which completely abrogates the pMHC1/CD8 interaction (12), failed to induce detectable amounts of fully phosphorylated ζ chain (Fig. 2A). We also examined the effects of antigen concentration, on the activation of CTL, by IFN γ ELISpot (Fig. 2B). Target cells expressing A245V-substituted HLA A2 were only marginally impaired in their ability to activate CTL despite a 5-fold reduction in their ability to interact with CD8 (Fig. 2B). In contrast, there was a significant reduction in activation by C1R targets expressing D227K/T228A HLA A2 (Fig. 2B). These data indicate that the CD8 T cell coreceptor maintains the majority of its biological activity even at extremely low binding affinities. To confirm that this result was affinity related, and not a peculiarity of structural conformation, we tested the biological effects of other mutations that similarly reduced the pMHC1/CD8 interaction.

Multimerized wild type HLA A2/ β_2m , HLA A2/K58E β_2m , and D227K/T228A HLA A2/ β_2m all bound to cell surface TCR equally well despite differences in the pMHC1/CD8 interaction (Fig. 3A). This is consistent with previous work showing that HLA A2 wild type and CD8 null tetramers exhibit comparable binding to cell surface TCR even at low concentrations (12). We noticed that if our pMHC1 tetramers were kept at 4°C for prolonged periods of time, the staining intensity of those manufactured with the D227K/T228A-mutated HLA A2 heavy chain was less than for those manufactured with the wild type heavy chain (Fig. 3B). It therefore appears that the biotinylated D227K/T228A mutated molecule is marginally less stable at 4°C than wild type molecules.

We have shown that multimeric forms of pMHC1 can induce an early signaling cascade almost identical to that produced by the cell surface presentation of antigen and that this signaling cascade results in effector function (12). Here, we examined the TCR-mediated early signal transduction cascade induced by different multimeric forms of pMHC1. Multimerized wild type HLA A2/ β_2m and HLA A2/K58E β_2m both induced a good in-

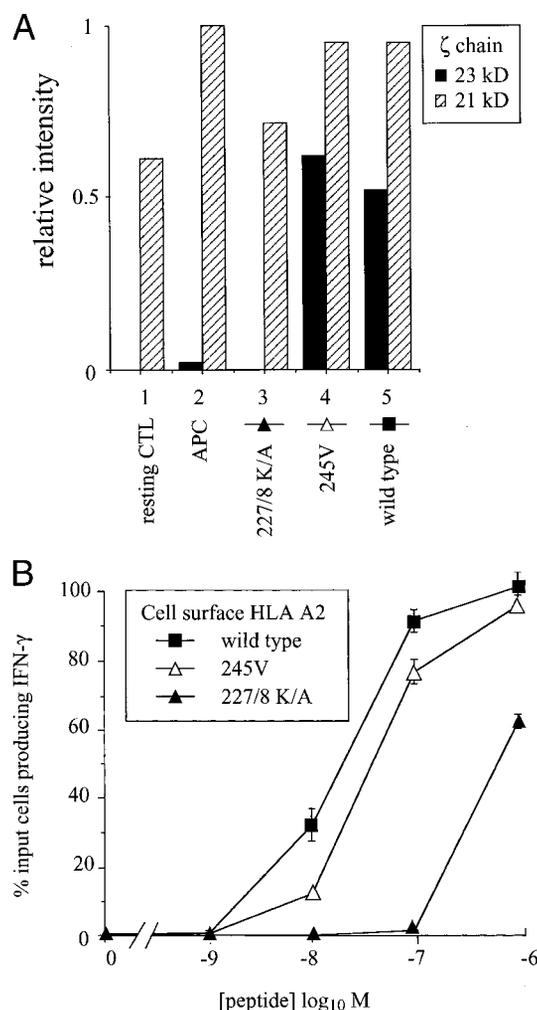


FIG. 2. CD8 maintains function at low binding affinities. *A*, induction of early signal transduction by C1R cells stably transfected with HLA A2. Lysates were run on SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody as described under "Experimental Procedures." Data was collected by FluorSMax (Bio-Rad). Relative intensities of the 23-kDa fully phosphorylated form and the 21-kDa partially phosphorylated form of CD3 ζ -chain (67) are shown. C1R transfectants were pulsed with the HLA A2-restricted HIV *gag*-derived epitope SLYNTVATL at a concentration of 1 μ M for 1 h and then washed 3 times. Peptide-pulsed C1R transfectants were presented to CTL clone 003 (68) for 10 min. *Bars 1*, resting CTL (no antigen presenting cells); *2*, CTL exposed to unpulsed C1R cells expressing wild type HLA-A2; *3*, CTL exposed to D227K/T228A HLA A2-expressing C1R cells pulsed with 1 μ M peptide; *4*, CTL exposed to A245V HLA A2-expressing C1R cells pulsed with 1 μ M peptide; *5*, CTL exposed to C1R cells expressing wild type HLA-A2 pulsed with 1 μ M peptide. Equal lane loading was confirmed as described under "Experimental Procedures." *B*, IFN- γ release by 003 CTL in response to C1R cells expressing wild type HLA A2, D227K/T228A HLA A2, or A245V HLA A2. C1R transfectants pulsed with SLYNTVATL were used as targets for the SLYNTVATL-specific CTL clone 003 in a 4-h ELISpot assay.

tracellular signal including full phosphorylation of the TCR ζ chain (Fig. 4A). Multimerized HLA A2 D227K/T228A failed to induce an early signal (Fig. 4A) despite being equal to wild type HLA A2 in terms of cross-linking cell surface TCR. A similar pattern (HLA A2/ β_2m = HLA A2/K58E β_2m \gg D227K/T228A HLA A2/ β_2m) was observed when multimer-induced RANTES, MIP1 β , and IFN- γ production were examined over a range of multimer concentrations (Fig. 4, *B–D*). Thus, HLA A2 folded with peptide and a K58E variant of β_2m can retain the vast majority of CD8-dependent functional activity despite a greater than 15-fold reduction (Fig. 1) in CD8 binding.

Naturally Weakened pMHC1/CD8 Coreceptor Interactions Maintain Biological Function—Previous studies have indi-

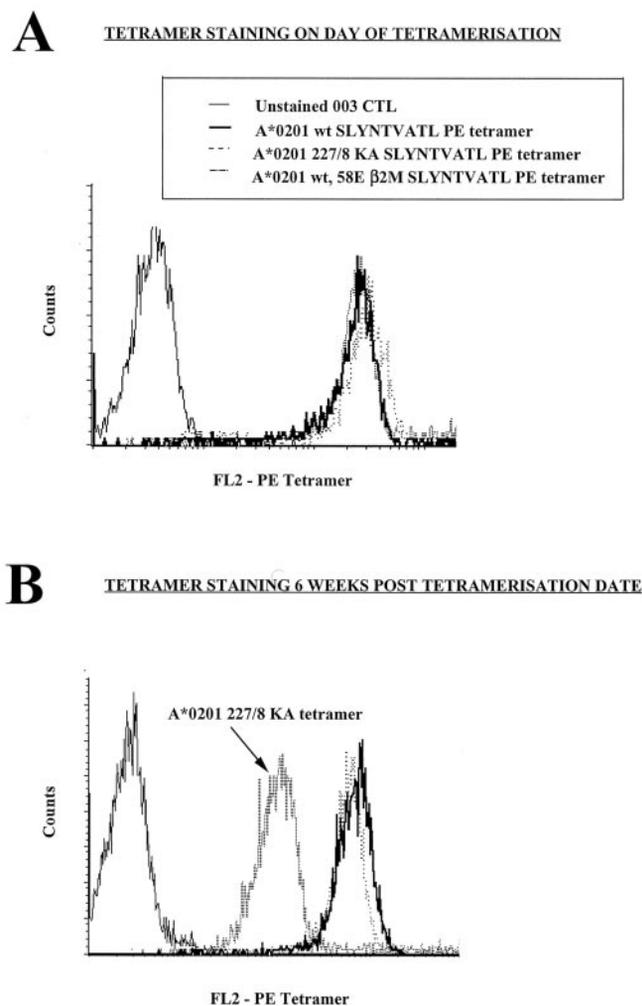


FIG. 3. D227K/T228A HLA A2 α 3 domain-mutated multimeric complexes are unstable compared with wild type molecules. A, 1×10^5 003 CTL in 20 μ l of PBS were stained with 1 μ g (pMHC I content) of SLYNTVATL-HLA A2/ β_2 m, SLYNTVATL-HLA A2/K58E β_2 m, or SLYNTVATL-D227K/T228A HLA A2/ β_2 m multimeric complexes. Multimeric complexes had been conjugated immediately prior to staining by addition of a phycoerythrin (PE)-extravidin conjugate (Sigma) to biotinylated monomeric complexes produced as described previously at a pMHC I:extravidin molar ratio of 4:1. B, stock solutions of all 3 tetrameric complexes used in A were stored under identical conditions (4 $^{\circ}$ C) for 6 weeks and staining subsequently repeated as above.

cated that HLA A68 fails to bind CD8 (45) and that the activation of HLA A68 CTL is completely independent of the pMHC I/CD8 interaction (46). Molecular modeling based on the structures of HLA A2, HLA A68, and the HLA A2/CD8 cocystal predict that the larger valine residue at position 245 in HLA A68 distorts the α 3 loop of the molecule resulting in a less energetically favorable interaction with CD8 (5). Our results (above) show that the A245V substitution in the α 3 domain of HLA A2 retains functional significance despite a 5-fold reduction in CD8 coreceptor binding affinity. We predicted that this level of interaction would remain functionally significant in HLA A68-restricted CTL. To test this, we manufactured HLA A68 and D227K/T228A HLA A68 as described under "Experimental Procedures." This double substitution knocks out the CD8 interaction with HLA A2 (12). We examined the binding of sCD8 to HLA A68 and D227K/T228A HLA A68 and V245A HLA A68 by SPR. In contrast to a previous report (45), we found that sCD8 does indeed bind to HLA A6801 (Fig. 5). As predicted (5), the binding was of lower affinity than that of

other classical MHC class I molecules ($K_D = 980 \mu$ M). The D227K/T228A variant of HLA A68 failed to bind sCD8 (Fig. 5).

We then tested the biological relevance of the naturally weak HLA A68/CD8 interaction when antigen is presented on the surface of antigen presenting cells. C1R cells expressing HLA A68 and D227K/T228A HLA A68 were produced as described under "Experimental Procedures." These cells were shown to express similar levels of MHC class I on their surface by staining with MHC class I specific antibody (Fig. 6A). C1R HLA A68 and C1R HLA D227K/T228A A68 were used to present antigen to HLA A68-restricted CTL specific for a HIV-1 Tat epitope (ITKGLGISYGR) (37). D227K/T228A HLA A68-expressing cells were impaired in their ability to activate a HLA A68-restricted, HIV-1 ITKGLGISYGR-specific CTL clone (c23) (Fig. 6, B and C). Importantly, multimeric forms of HLA A68 and D227K/T228A HLA A68 folded with ITKGLGISYGR peptide bound equally well to the cell surface of a CTL line that recognized this antigen thus ruling out any major difference in the α 1/ α 2 TCR-binding platform of these molecules (data not shown). Thus, HLA A68-restricted T cells do exhibit some dependence on the pMHC I/CD8 interaction.

DISCUSSION

Recognition of antigen by CTL is determined by interaction of both the TCR and its CD8 coreceptor with pMHC I molecules. Four possible roles for CD8 in T cell activation can be envisaged (24, 47). First, CD8 performs a role in TCR signal transduction (12). After CTL engage pMHC I, the earliest intracellular events induce specific phosphorylation of tyrosine residues in the immunoreceptor tyrosine activation motifs within the cytoplasmic tails of the TCR-associated TCR/CD3/ ζ 2 complex. The cytoplasmic tail of the CD8 α -chain is associated with the protein-tyrosine kinase p56^{lck} (8). Active p56^{lck} initiates TCR signal transduction by phosphorylating the immunoreceptor tyrosine activation motifs within the TCR/CD3/ ζ 2 complex. However, functional T cell activation, including p56^{lck} activation, can be achieved without involving pMHC I/CD8 contacts, for example, with monoclonal antibodies against components of the TCR/CD3/ ζ 2 complex (48, 49). Furthermore, it remains unclear whether T cell activation by physiological levels of pMHC I antigen always requires signaling through the coreceptor or whether this function is dispensable under certain conditions (48, 50). Second, the co-receptor may have a role in assisting cell-cell adhesion, helping to tether the T cell to the antigen presenting cell (51). Third, CD8 may assist the TCR/pMHC I interaction by binding cooperatively with the TCR to the same pMHC I molecule (52). Fourth, CD8 may play a role in the activation of CTL via direct interaction with the TCR (53, 54), rather than pMHC I. Recent evidence suggests that the TCR/CD8 interaction may favorably influence the distribution of the TCR on the CTL surface (24). Cooperative binding of TCR and CD8 to a single pMHC I molecule has been ruled out by structural (5) and biophysical (24, 32) observations. However, it remains unclear whether the dominant effect of the pMHC I/CD8 interaction in the activation of human CTL is a direct consequence of the binding energy this interaction affords or the role of this extracellular interaction in the delivery of signaling molecules to the cytoplasmic side of the TCR/CD3/ ζ 2 complex.

The study of the pMHC I/CD8 interaction has been confounded by a number of differences in the systems and methodologies utilized by different groups. First, we have recently shown that the human pMHC I/CD8 interaction is 4 times weaker than the equivalent murine interaction in the absence of glycosylation (12). There are no reported differences in the TCR/pMHC I interactions of these two species and it is likely that any pMHC I/CD8 binding effects will be more prominent in

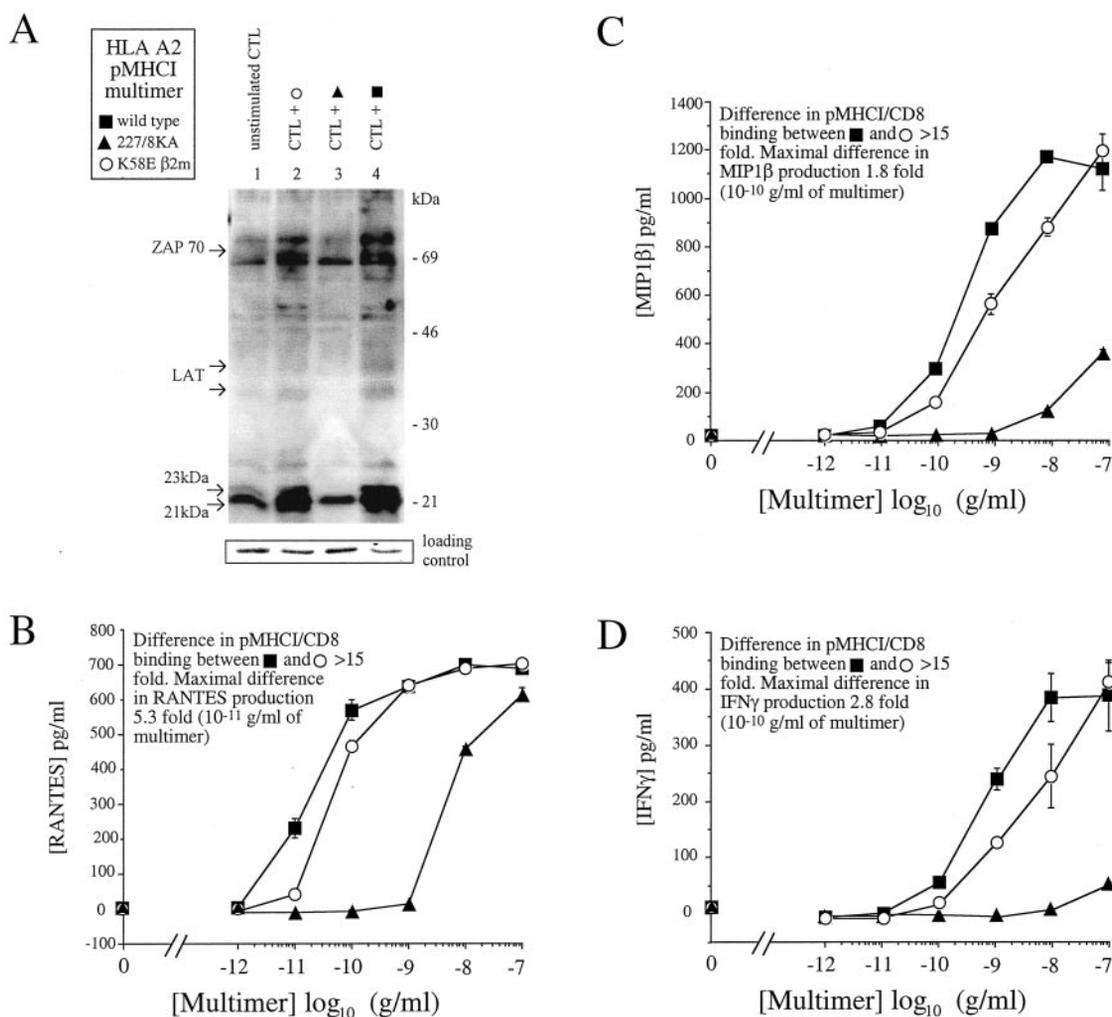


FIG. 4. Activation of HLA A2-restricted CTL with multimeric pMHC I with altered CD8 coreceptor interactions. A, activation of early TCR signal transduction in response to wild type HLA A2 (filled squares), D227K/T228A HLA A2 (filled triangles), or K58E/ β_2m HLA A2 multimers (open circles). 003 CTL (10^6 /lane) were stimulated for 3 min, and cell lysates were immunoblotted with anti-phosphotyrosine monoclonal antibody as described. Lane 1, resting CTL presented with no stimulus; lane 2, CTL stimulated with 1 μ g/ml SLYNTVATL-HLA A2/K58E/ β_2m multimer for 3 min; lane 3, CTL stimulated with 1 μ g/ml SLYNTVATL-D227K/T228A HLA A2/ β_2m multimer for 3 min; lane 4, CTL stimulated with 1 μ g/ml SLYNTVATL-wild type HLA A2/ β_2m multimer for 3 min. B–D, soluble lymphokine release by 003 CTL in response to wild type HLA A2/ β_2m , D227K/T228A HLA A2/ β_2m , or HLA A2/K58E/ β_2m multimers, all bearing the SLYNTVATL peptide. Lymphokine release was determined by ELISA 4 h after addition of stimulus as previously described (69). Graphs show the mean \pm S.D. of two replicate assays. Staining levels determined by flow cytometric quantification were equivalent with all 3 multimers. Plots are for RANTES (B), MIP1 β (C), and IFN- γ (D) levels in the same supernatant. Lymphokine released in response to the wild type HLA A2/ β_2m and HLA A2/K58E/ β_2m multimers differed most at the lowest antigen concentrations. These differences are indicated on the plots along with the difference in the pMHC I/CD8 binding of these two multimers.

the mouse rather than the human systems studied here. Second, it has been shown that the affinity of the pMHC I/CD8 varies for different human (34) and murine (55) MHC class I molecules. Third, recent evidence shows that the glycosylation of CD8 can also affect the binding to MHC I (30, 31). Consequently, the effects of CD8 are likely to vary depending on the species, T cell, and particular MHC I molecule being studied. The picture has been further blurred by the use of anti-CD8 antibodies to study the pMHC I/CD8 interaction. We have recently shown that human anti-CD8 “blocking” antibodies block T cell activation, and staining with multimeric pMHC I, by interfering with the TCR/pMHC I interaction and not, as previously assumed, the pMHC I/CD8 interaction.³ Similar findings have recently been reported in the mouse (56). The use of anti-CD8 antibodies to examine the role of CD8 in CTL activation does not allow discrimination between the interaction of

CD8 with pMHC I on the surface of the target cell, interaction with the TCR on the CTL surface, or other possible roles for CD8.

Previous studies have examined the roles of the extracellular binding and cytoplasmic lck recruitment components of the CD8 molecule in murine hybridomas (57, 58). Comparison of CD8⁻ T cell hybridomas transfected with CD8 α and tailless CD8 α' molecules indicate that both can aid interleukin-2 production, although restoration by CD8 α' is minimal (about 10% of the wild type molecule). The use of tailless CD8 molecules also suffers from the same caveat as the use of anti-CD8 antibodies in that it does not allow discrimination between the interaction of CD8 with pMHC I on the surface of the target cell, interaction with the TCR on the CTL surface, or other possible roles for CD8. As other roles of the external domain of CD8 were not appreciated at the time of these murine hybridoma studies it was believed that the increases in activation afforded by tailless CD8 molecules were “presumably achieved by increasing the avidity of the TCR-antigen interaction through binding of CD8 to class I molecules” (58). Due to the inability of

³ L. Wooldridge, S. L. Hutchinson, E. M. Choi, A. Lissina, E. Jones, F. Mirza, P. R. Dunbar, D. A. Price, V. Cerundolo, and A. K. Sewell, manuscript in preparation.

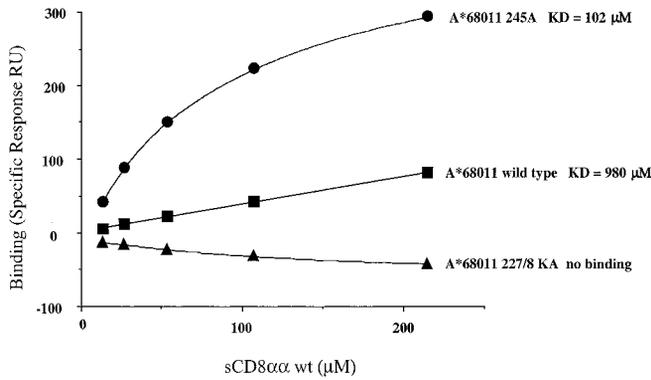


FIG. 5. The affinity of sCD8 α binding to HLA-A68/ β_2 m monomers. ITKGLGISYGR-HLA A68 (squares), ITKGLGISYGR-D227K/T228A HLA A68 (triangles), and ITKGLGISYGR-V245A HLA A68 (circles) were loaded onto separate flow cells of a streptavidin-coated research grade CM5 chip (BLAcore AB) at \sim 1000 response units (RU). A third flow cell was loaded with biotinylated sTCR, specific for the HLA A2-restricted HLTV-1 Tax epitope LLFGYPVYV at 1000 RU, to act as a negative control. sCD8 α was injected at increasing concentrations through all 4 flow cells. For each concentration of sCD8 α (μ M) the specific binding was calculated as the difference between the responses at equilibrium in the experimental flow cells compared with the negative control. Specific binding was then plotted against the sCD8 α concentration for non-linear curve analysis. Linear Scatchard plot analysis was also performed (data not shown).

anti-CD8 antibodies or the use of tailless CD8 molecules to distinguish between different roles of the extracellular domain of CD8 we believe the most desirable way to examine the role of the pMHC/CD8 interaction in isolation is to utilize mutations in pMHC that affect the pMHC/CD8 interaction without altering the TCR/pMHC interaction (12, 36). In this study we have used mutations in the α 3 domain of the MHC heavy chain and the β_2 m subunit that reduce the pMHC/CD8 interaction to probe the significance of pMHC/coreceptor affinity in the activation of human CTL.

We find that the CD8 coreceptor exhibits disproportionate biological activity at extremely low binding affinities using several different systems. First we demonstrated that HLA A2 retains the vast majority of its CD8-dependent functional activity when residue 245 is mutated to valine (Fig. 2). This mutation, which occurs naturally in HLA A68, reduces the binding of CD8 to HLA A2 by 5-fold (34). We then utilized the K58E mutation in β_2 m. This mutation has a more profound effect on the binding of sCD8. The $>$ 15-fold reduction in CD8 binding caused by the K58E mutation in β_2 m translated into, at most, a 5.3-, 1.8-, and 2.8-fold reduction in RANTES, MIP1 β , and IFN γ production, respectively (Fig. 4, B–D). Our results show that CD8 maintains the vast majority of its activity even when the pMHC/CD8 interaction is extremely weak (Fig. 2 and 4). Consequently, we favor a model where the dominant effect of CD8 in the activation of human CTL is not a direct consequence of the binding energy provided by the extracellular interaction between pMHC and CD8. Finally, we examined the role of CD8 in the activation of HLA A68-restricted T cells by introducing the D227K/T228A mutation into the α 3 domain of MHC class I does not affect the binding of TCRs to the α 1/ α 2 peptide-binding platform of the HLA A2 molecule (12); there is no reason to assume that this would not be the case with HLA A68. We studied the structure of the human class I histocompatibility antigen Aw68.1 solved at 1.9-Å resolution and deposited in the Protein Data Bank (59) as 1hsb (60). The distances between the C α atoms of residues Asp-227, Thr-228, and Val-245 and the centroid of the presented peptide were 46.3 Å, 43.2 Å, and 39.6 Å, respectively. These extremely

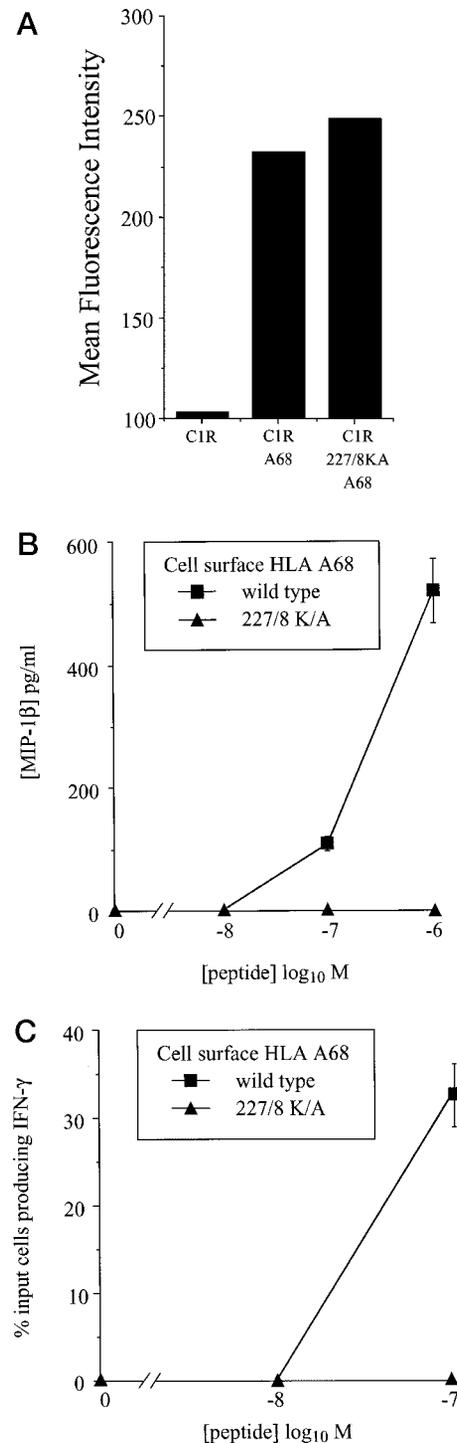


FIG. 6. HLA A68-restricted CTL exhibit dependence on the pMHC/CD8 interaction. A, expression of HLA A68 and D227K/T228A HLA A68 on the surface of retrovirally transduced C1R cells quantified by flow cytometry. 10^6 cells were prepared and stained with 1 μ g of anti-HLA class I (W6/32) antibody for 30 min on ice as described under “Experimental Procedures.” The data are representative of three separate experiments. B, CD8-dependent release of MIP-1 β by c23 CTL stimulated with ITKGLGISYGR-presenting C1R cells expressing wild type HLA A68 (squares) or D227K/T228A HLA A68 (triangles). MIP-1 β release was determined at 4 h by ELISA. C, release of IFN- γ by c23 CTL stimulated with ITKGLGISYGR-pulsed C1R cells expressing wild type HLA A68 (squares) or D227K/T228A HLA A68 (triangles). IFN- γ release was determined at 4 h by ELISpot.

large distances support the hypothesis that mutating Asp-227, Thr-228, and Ala-245 residues would be unlikely to affect the α 1/ α 2 peptide-presenting platform. Furthermore, multimeric

forms of HLA A68 and D227K/T228A HLA A68 folded with the ITKGLGISYGR peptide bound to CTL at equivalent levels (data not shown), thus, providing experimental support for this prediction. Previous studies have reported that HLA A68 does not bind to CD8 and that A68-restricted CTL are completely independent of the pMHCI/CD8 interaction (45, 46). In keeping with the results above, however, we found that: (i) the natural mutation that occurs in the $\alpha 3$ domain (A245V) of HLA A68 does bind CD8, albeit with a markedly reduced affinity ($K_D = 980 \mu\text{M}$; Fig. 5), and (ii) this HLA A68/CD8 interaction is functionally significant in the natural setting (Fig. 6). Indeed, the very existence of HLA A68-restricted CTL is evidence, in itself, of a functional HLA A68/CD8 interaction. CD8-deficient mice fail to develop CTL suggesting a role for the pMHCI/CD8 interaction in thymic selection (61). This has been confirmed using transgenic MHCI molecules impaired in their interaction with CD8 (62–65). Whereas there are differences in the pMHCI/CD8 interaction between mouse and human (66), it is reasonable to assume that this interaction is also important for the development of human CTL. There is no evidence that HLA A68-positive individuals are impaired in the development of A68-restricted CTL. Thus, the reduced affinity of this molecule for CD8 appears sufficient for T cell development in addition to having functional significance in the periphery.

It should be noted that the energy of the pMHCI/CD8 interaction still plays a role in the activation of human antiviral CTL. Changes in the dose-response curve generated by antigen with extremely weak pMHCI/CD8 interactions show that the binding energy of this interaction has significant effects, particularly at very low antigen concentrations (Figs. 2B and 4, B–D). Whereas these decreases in CTL sensitivity are not in proportion to the decreases in pMHCI/CD8 binding, they demonstrate that the binding energy of this interaction plays a role in enhancing the sensitivity of human anti-viral CTL. The increase in sensitivity afforded by this binding energy may be particularly relevant *in vivo* where antigen is likely to be limiting.

The affinities of the pMHCI/CD8 and pMHCII/CD4 interactions are extremely low ($K_D > 100 \mu\text{M}$) (32–34). Cell-cell adhesion molecules that interact with 1:1 stoichiometry typically do so with higher affinity (7, 32). The extraordinarily low affinity of the pMHC/coreceptor interaction is unlikely to be the result of an evolutionary accident. We suggest that the uniquely low affinity of the pMHC/coreceptor interaction is critical for T cell specificity. The binding sites for CD8 and CD4 are separate from the TCR-recognized (2), peptide-binding domains of MHC molecules, and allow a single MHC molecule to be bound simultaneously by both TCR and CD8 or CD4 (5, 6). The highly variable complementarity determining regions of the TCR, which interact with the peptide-binding platform of pMHCI, confer antigen specificity to the T cell recognition process (1, 2). We have measured the human TCR/pMHCI interaction for 12 TCR/ligand pairs⁴ and observe a median K_D of $\sim 10 \mu\text{M}$. We have also measured equilibrium binding of pMHCI and CD8 for a variety of different HLA gene products and find that it has a K_D in excess of $100 \mu\text{M}$ (34). Thus, the human TCR/pMHCI interaction is generally 10-fold higher than the pMHCI/CD8 interaction. As it is the highly variable TCR that determines the specificity of pMHC recognition, it is essential that the affinity of the TCR/pMHC interaction dominates the interaction of pMHC with the invariant coreceptor in TCR/pMHCI/CD8 or TCR/pMHCII/CD4 interactions. We predict that an increased interaction between the pMHC and coreceptor would compromise T cell specificity. Experiments to test this hypothesis are currently in progress.

In summary, we have shown that the pMHCI/CD8 interaction can maintain substantial biological activity at extremely low binding affinities. Whereas our findings show that the binding energy of the pMHCI/CD8 interaction has some biological significance it does not represent the dominant role of the CD8 coreceptor in the activation of natural anti-viral human CTL. The binding energy provided by the pMHCI/CD8 interaction may play a more significant role in the activation of murine CTL, or human CTL with weak TCR/pMHCI interactions. However, the dominant role for the extracellular pMHCI/CD8 interaction in the activation of the human anti-viral CTL studied here appears to be the delivery of signaling molecules to the cytoplasmic side of the TCR/CD3/ ζ_2 complex.

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