

The Human CD8 Coreceptor Effects Cytotoxic T Cell Activation and Antigen Sensitivity Primarily by Mediating Complete Phosphorylation of the T Cell Receptor ζ Chain*

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Recognition of antigen by cytotoxic T lymphocytes (CTL) is determined by interaction of both the T cell receptor and its CD8 coreceptor with peptide-major histocompatibility complex (pMHC) class I molecules. We examine the relative roles of these receptors in the activation of human CTL using mutations in MHC class I designed to diminish or abrogate the CD8/pMHC interaction. We use surface plasmon resonance to determine that point mutation of the $\alpha 3$ loop of HLA A2 abrogates the CD8/pMHC interaction without affecting the affinity of the T cell receptor/pMHC interaction. Antigen-presenting cells expressing HLA A2 which does not bind to CD8 fail to activate CTL at any peptide concentration. Comparison of CTL activation by targets expressing HLA A2 with normal, abrogated, or diminished CD8/pMHC interaction show that the CD8/pMHC interaction enhances sensitivity to antigen. We determine that the biochemical basis for coreceptor dependence is the activation of the 23-kDa phosphoform of the CD3 ζ chain. In addition, we produce mutant MHC class I multimers that specifically stain but do not activate CTL. These reagents may prove useful in circumventing undesirable activation-related perturbation of intracellular processes when pMHC multimers are used to phenotype antigen-specific CD8+ lymphocytes.

T lymphocytes recognize peptide antigen associated with major histocompatibility complex (MHC)¹ molecules on the surface of antigen-presenting cells (APCs). The antigen specificity of T lymphocytes is conferred by the T cell receptor (TCR), whose highly variable complementarity-determining regions interact with the $\alpha 1/\alpha 2$ domain platform of the MHC and the bound peptide. Engagement of the TCR with peptide-MHC (pMHC) activates an intracellular signal transduction cascade

via the TCR-associated chains of the CD3- ζ_2 complex. The signal from the TCR ultimately leads to activation of the various T cell effector functions.

In addition to its role in presenting antigen to the TCR, pMHC also interacts with the T cell coreceptors CD4 and CD8, which bind to invariable regions of MHC class II and MHC class I molecules, respectively. These coreceptors are critical for the development and activation of most T cells. Three possible roles for the coreceptor in T cell activation can be envisaged (1). First, the coreceptors perform a role in TCR signal transduction. After T cells engage pMHC, the earliest intracellular events induce specific phosphorylation of tyrosine residues in the immunoreceptor tyrosine activation motifs within the cytoplasmic tails of the TCR-associated CD3 complex. The cytoplasmic tails of CD4 and the CD8 α -chain are associated with the protein tyrosine kinase p56^{lck}. Active p56^{lck} initiates TCR signal transduction by phosphorylating the immunoreceptor tyrosine activation motifs within the CD3 complex. However, functional T cell activation, including p56^{lck} activation, can be achieved without involving coreceptor-MHC contacts, for example with monoclonal antibodies against components of the TCR-CD3 complex (2, 3). Furthermore, it remains unclear whether T cell activation by physiological levels of pMHC antigen always requires signaling through the coreceptor or whether this function is dispensable under certain conditions (4–6). Second, the co-receptor may have a role in assisting cell-cell adhesion, helping to tether the T cell to the APC (7). Third, the co-receptor may assist the TCR-MHC interaction by binding cooperatively with the TCR to the same pMHC molecule (8). For the murine MHC class I antigen recognition complex, surface plasmon resonance (SPR) suggests that binding of soluble CD8 $\alpha\alpha$ and TCRs to immobilized pMHC class I decreases the dissociation-rate of the TCR/pMHC interaction by approximately 7-fold (9). However, it is difficult to envisage how this cooperativity might arise in the human TCR/pMHC interaction. Comparison of the structures of the human TCR-HLA A2 and CD8-HLA A2 complexes (10) shows that binding of the CD8 $\alpha\alpha$ homodimer to HLA A2 does not transmit structural changes to the TCR binding site (10), suggesting that formation of the human CD8-MHC-TCR complex is not cooperative. SPR measurements confirm this notion and do not indicate an increase in binding of human TCRs to HLA A2 in the presence of human CD8 $\alpha\alpha$ (11).

In this study, we use cell surface expressed and soluble multimeric pMHC complexes mutated to alter the CD8/pMHC interaction to evaluate the role of the CD8 coreceptor in the activation of human CTL.

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¹ The abbreviations used are: MHC, major histocompatibility complex; APC, antigen-presenting cell; TCR, T cell receptor; pMHC, peptide-major histocompatibility complex; SPR, surface plasmon resonance; CTL, cytotoxic T lymphocytes; HIV-1, human immunodeficiency virus type 1; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; PE, phycoerythrin.

EXPERIMENTAL PROCEDURES

Cell Lines and Clones—The HLA A2 HIV-1 Gag p17-8 (SLYNTVATL)-specific CTL were cultured from the peripheral blood mononuclear cells of HIV-1-infected patients 868 and 003 as previously described (12). The melanoma-specific CTL clone 3G10 has been described elsewhere (13). B cells were transformed by infecting 5×10^6 peripheral blood mononuclear cells with 1 ml of supernatant from Epstein-Barr virus-infected B95-8 cells (14).

Immunoblotting—Cells were pelleted and resuspended vigorously in 20 ml of lysis buffer (10% glycerol, 1% Nonidet P-40, 140 mM NaCl, 20 mM Tris, pH 8.0, 10 mM NaF, 2 mM EDTA, 1 mM sodium orthovanadate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin). Nuclei were pelleted (15 min at 13,000 rpm), and supernatants were boiled for 5 min after the addition of 5 μ l of reducing buffer (125 mM Tris, pH 6.6, 20% glycerol, 10% β -mercaptoethanol, 4% SDS). Proteins were separated overnight by SDS-polyacrylamide gel electrophoresis on 12% acrylamide and subsequently transferred onto Hybond-ECL nitrocellulose (Amersham Pharmacia Biotech) by semidry Western blotting. Blots were blocked for 2 h at 4 °C with 1% bovine serum albumin in PBS-Tween (0.1% Tween 20 in PBS) and probed overnight with anti-phosphotyrosine monoclonal antibody 4G10 at 4 °C (Upstate Biotechnology, Inc.; 1 μ g/ml, 0.1% bovine serum albumin in PBS-Tween). Peroxidase-linked anti-mouse antibody (Amersham Pharmacia Biotech) was used as secondary antibody and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). To control for protein loading, blots were re-probed to detect total cellular ZAP 70. To reprobe blots, membranes were left at 4 °C for at least 48 h to allow peroxidase to inactivate. Membranes were rehydrated in PBS-Tween and incubated with anti-ZAP 70 polyclonal antibody derived from goat (Serotech; 1:1000 in 2.5% milk powder/PBS-Tween) overnight at 4 °C. Blots were washed 3 times for 10 min in PBS-Tween and subsequently incubated with peroxidase-linked anti-goat secondary antibody (Serotech; 1:1000 dilution in 2.5% milk powder/PBS-Tween) for 90 min at 4 °C. Membranes were washed 3 times for 15 min with PBS-Tween, and peroxidase activity was detected by ECL. For activation of CTL by APC, peptide-pulsed Epstein-Barr virus-transformed B cell lines (14) were presented to CTL at a ratio of 1:10 in a final volume of 50 μ l (15).

MHC Class I Multimers—Biotinylated peptide-HLA A2 complexes were prepared as previously described (16). HLA A2 was multimerized by the addition of PE-conjugated streptavidin. Concentration of pMHC multimers as expressed throughout refers only to the pMHC component.

Transfection of C1R Cells—C1R cells (17) were cultured in RPMI 1640 supplemented with glutamine and 10% fetal calf serum. C1R cells were transfected by electroporation (Bio-Rad), and transfectants were selected by culturing cells in 1 mg/ml G418 sulfate 48 h after electroporation. Transfectants were stained with conformation-specific anti-HLA A2 antibody BB7.2 (18) and sorted by fluorescence-activated cell sorting to pool the fraction of cells expressing the highest levels of HLA A2. Clones were established and stained with BB7.2 to select clones expressing similar levels of wild type or mutant HLA A2. We have previously examined the binding to MHC of the HLA A2-restricted influenza matrix epitope GILGFVFTL, the HIV-1 Gag-derived epitope SLYNTVATL, and 10 natural variants of this peptide using a conventional stabilization assay (12) and cell surface stabilization by fluorescence-activated cell sorting (as in Ref. 19). The relative binding efficiencies obtained correlate well with the efficiency of HLA A2 refolding in solution (data not shown). Thus, relative stabilization in solution (refolding) gives similar results to relative stabilization (peptide loading) on the cell surface. Since wild type, A245V, and D227K/T228A HLA A2 stably refold around the YMDGTMSQV melanoma peptide to an identical extent in solution during tetramer production, equal peptide-loading efficiency of these molecules when transfected and expressed on cell surface is implicit.

Production of Soluble TCR and CD8—Soluble human TCR and CD8 $\alpha\alpha$ were prepared as described before (11, 20–22). Briefly, all proteins were expressed in the form of insoluble inclusion bodies in *Escherichia coli* strain BL21(DE3)pLysS from a plasmid containing the gene encoding the protein under the control of a T7 promoter. Inclusion bodies were released from *E. coli* by sonication and purified by washing with buffer containing 0.5% Triton X-100. Prior to refolding, protein was solubilized in a buffer containing 6 M guanidine HCl and 10 mM dithiothreitol. Refolding was initiated by rapid dilution of protein into refolding buffer containing 0.4 M L-arginine-HCl and a redox couple (3.7 mM cystamine, 6.6 mM β -mercaptoethylamine) to a concentration of 60 mg/liter. In the case of soluble TCR, the refolding buffer also contained

5 M urea, and in the case of pMHC complexes, the refolding buffer also contained 4 mg/liter synthetic peptide. Proteins were purified using standard column chromatography techniques. Human CD8 was first dialyzed against 10 mM MES, pH 6.0, and then purified by cation exchange chromatography on a POROS 50 HS column, followed by gel filtration chromatography on an Amersham Pharmacia Biotech Superdex 75 HR column. Human TCR was dialyzed against 10 mM Tris, pH 8.1, and then purified by anion exchange chromatography on a POROS 50 HQ column, followed by gel filtration chromatography on an Amersham Pharmacia Biotech Superdex 200 HR column. Mouse CD8 was prepared as for human CD8. Human pMHC complexes were dialyzed against 10 mM Tris, pH 8.1, and then purified by anion exchange on a POROS 50 HQ column. Mouse pMHC complexes were concentrated using 10-kDa cut-off membranes and purified by gel filtration chromatography on an Amersham Pharmacia Biotech Superdex 75 HR column. Both human and mouse pMHC complexes were expressed with a biotinylation tag on the C terminus and were biotinylated by incubation with BirA enzyme in the presence of Mg-ATP and biotin according to the method of Ref. 16. Complexes were purified by gel filtration chromatography using an Amersham Pharmacia Biotech Superdex 75 HR column.

Surface Plasmon Resonance—BIAcore biomolecular interaction analysis was performed using a BIAcore 3000™ machine equipped with a CM-5 sensor chip, which was modified with streptavidin using standard amine coupling, as previously described (11, 20, 23). Complexes were immobilized on the surface of individual cells to a concentration of ~11,000 response units. After immobilization of the complexes, protein solutions were flowed over the cells in series at varying concentrations, and data were recorded. The data were analyzed with the BIAeval program, Excel, and Microcal Origin. K_D values were calculated, assuming 1:1 Langmuir binding ($A + B \leftrightarrow AB$), using nonlinear curve fitting to the following equation: $AB = B \cdot AB_{max} / (K_D + B)$.

RESULTS

Mutations in the $\alpha 3$ Domain of HLA A2 Inhibit CD8/HLA A2 Interaction without Affecting TCR/HLA A2 Interaction—MHC class I molecules interact with both the TCR and its CD8 coreceptor. To dissect the contribution of the CD8/pMHC interaction to T cell activation from that of the TCR/pMHC interaction, we mutated two residues (D227K and T228A) in the $\alpha 3$ domain of HLA A2 that appear to be important for the interaction with CD8 (10). SPR analysis showed that TCR binds with equal affinity to wild type HLA A2 and to the D227K/T228A mutant complexes (Fig. 1). Conversely, soluble CD8 $\alpha\alpha$ binds only to wild type HLA A2 but not to D227K/T228A HLA A2 (Fig. 1). The D227K/T228A mutations thus inhibit the CD8/pMHC class I interaction without affecting the TCR/pMHC class I interaction.

D227K/T228A HLA A2 Multimers Stain CTL Efficiently—It has been suggested that the coreceptor stabilizes the interaction between pMHC and TCR. Multimeric forms of pMHC stably cohere to the T cell surface in an antigen-specific manner (24, 25) and are capable of interacting with both cell surface TCR and CD8.

We have extensively characterized CTL from donors 003 and 868, which are specific for the HLA A2-restricted HIV-1 Gag p17-8 epitope (SLYNTVATL) (12, 15, 26). We stained 003 and 868 CTL with multimeric complexes of SLYNTVATL-wild type HLA A2 and SLYNTVATL-D227K/T228A HLA A2 to determine the contribution of CD8 to the extent and rate of pMHC adhesion to antigen-specific T cells (Fig. 2). Both wild type and D227K/T228A mutant pMHC multimers stain 868 and 003 CTL with equal efficiency at 37 °C (Fig. 2A) and at 0 °C (data not shown). Furthermore, both multimers also stained CTL with similar intensity and at the same rate, achieving near maximal binding after only 5 min. Results for 003 CTL are shown in Fig. 2B. Finally, the staining intensity with wild type and D227K/T228A mutant multimers titrated between 1 μ g and 10 ng of multimer (Fig. 2C), demonstrating a similar intensity of staining with both multimers even when used at low concentration.

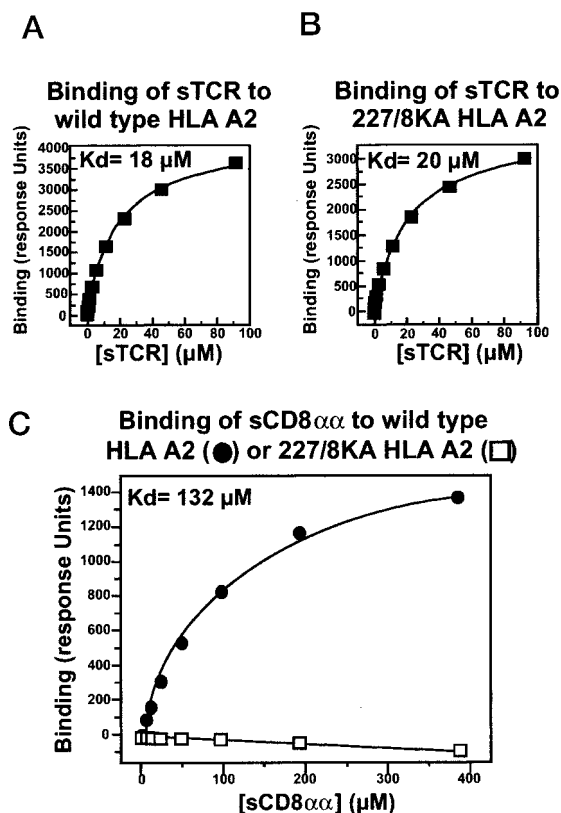


FIG. 1. Determining the binding affinities of wild type HLA A2 and D227K/T228A HLA A2 for TCR and CD8 $\alpha\alpha$. Biotinylated wild type HLA A2 or D227K/T228A HLA A2 presenting the influenza matrix protein-derived epitope of sequence GILGFVFTL was immobilized on a streptavidin-coated chip surface (BIAcore). The GILGFVFTL-specific TCR has been described previously (20, 21). Binding of soluble GILGFVFTL-specific HLA A2-restricted TCR (sTCR) or soluble CD8 $\alpha\alpha$ (sCD8 $\alpha\alpha$) to immobilized MHC was determined by SPR at 25 °C. *A*, binding of soluble GILGFVFTL-specific HLA A2-restricted TCR to wild type HLA A2. *B*, binding of soluble GILGFVFTL-specific HLA A2-restricted TCR to D227K/T228A HLA A2. *C*, SPR measurements to determine binding of sCD8 $\alpha\alpha$ to wild type HLA A2 (●) or D227K/T228A HLA A2 (□). K_d values were determined by linear least squares fitting to Scatchard plots.

D227K/T228A HLA A2 Multimers Activate CTL Poorly—

Since polyclonal cross-linking of the TCR can effect T cell activation, we used SLYNTVATL-wild type HLA A2 and SLYNTVATL-D227K/T228A HLA A2 multimers to investigate the contribution of CD8 to cellular activation (Fig. 3). The earliest biochemical events following TCR engagement with ligand are characterized by a series of protein tyrosine phosphorylation events that can be visualized in anti-phosphotyrosine immunoblots. We examined whether cross-linking of the TCR by soluble wild type pMHC multimers induced a normal pattern of early tyrosine phosphorylation by comparing this signal to that generated during physiological recognition (peptide presented on APC). Presentation of wild type pMHC class I multimers to 003 or 868 CTL induced tyrosine phosphorylation profiles almost identical to those induced by SLYNTVATL-bearing APC (Fig. 3A, 003 and 868 panels, lanes 2 and 3). This suggests that multivalent pMHC/TCR engagement results in the correct integration of the multiple cytoplasmic components involved in the early TCR signal transduction cascade. In contrast, multimerizing TCR with the D227K/T228A mutant pMHC multimer failed to induce the wild type profile of protein tyrosine phosphorylation events associated with antigen recognition in both 003 and 868 CTL (Fig. 3A). Several proteins critical for T cell activation (23-kDa CD3 ζ -chain and LAT) fail to become phosphorylated at all with the D227K/T228A mutant

pMHC multimer (Fig. 3A). Thus, the ability of pMHC multimer to interact with CD8 is essential for induction of the complete TCR signal transduction cascade. The use of mutant pMHC multimer excludes the possibility that activation seen with wild type pMHC multimer is due to free peptide. Further, the <10-kDa fraction of the multimer preparation, separated with a 10-kDa cut-off centricon (Amicon), failed to induce any tyrosine phosphorylation (data not shown).

Although CD8 appears critical for induction of the complete TCR-associated signal transduction cascade, we have previously shown that even a partial early signal can be sufficient to activate downstream CTL effector functions (12, 15). To determine the role of CD8 in induction of CTL effector functions, we compared the ability of wild type and D227K/T228A HLA A2 multimers to effect degranulation of CTL. We gauged degranulation by measuring the release of the CC chemokine RANTES, which is stored within the lytic granules of CTL (Fig. 3B) (27, 28). The failure of the D227K/T228A HLA A2 multimer to induce complete TCR signal transduction translates into a failure to induce activation of CTL at low multimer concentration as measured by RANTES release (Fig. 3B). However, this failure in activation was partially overcome by increasing the mutant multimer concentration by over 2 orders of magnitude (Fig. 3B). Consequently, activation can be achieved, despite an incomplete intracellular signal, by the cross-linking of TCR alone, provided that there is a substantial amount of antigen. This suggests that the ability of MHC class I to interact with CD8 has a dramatic effect on the sensitivity of CTL to antigen.

Reduced Activation of CTL by APC Expressing $\alpha 3$ Mutant HLA A2—

Although wild type pMHC class I multimers induce a physiological activation profile in CTL (Fig. 3A), such stimulation is still significantly removed from the *in vivo* situation, where antigen is presented as a minority species on the surface of an APC. In order to examine the requirement for CD8 in the recognition of APC by CTL, the HLA class I-deficient B cell line C1R (17) was transfected to express full-length HLA A2 or the CD8 nonbinding D227K/T228A HLA A2 mutant. Furthermore, C1R cells were transfected with a second mutant HLA A2 whose $\alpha 3$ domain was mutated at a single residue, A245V, to resemble the $\alpha 3$ domain of HLA A68. HLA A68 binds to CD8 with over 10-fold less affinity than does HLA A2 (23). The use of C1R cells transfected with wild type HLA A2, D227K/T228A A2, and A245V A2 enabled the antigen-specific recognition of targets with a normal, abrogated, and reduced CD8/pMHC interaction to be compared. This allowed us to examine the role of the CD8/pMHC class I interaction in sensitivity to antigen.

Cloned C1R transfectants were determined to express similar amounts of HLA A2 on the cell surface by fluorescence-activated cell sorting analysis using a conformation-specific HLA A2 antibody (data not shown). C1R transfectants were pulsed with the melanoma-derived HLA A2-restricted YMDGTMSQV epitope and presented to melanoma-specific CTL clone 3G10 (13) in a lysis assay. C1R cells transfected with HLA A2 were efficient targets for CTL in lysis assays (Fig. 4A), whereas the CD8-nonbinding D227K/T228A HLA A2 transfectants were not recognized efficiently at any peptide concentration tested. Additionally, recognition by CTL of C1R cells expressing the A68-like A245V HLA A2 was only achieved when cells were pulsed with peptide at between 1 and 2 orders of magnitude higher concentration than was sufficient for recognition of wild type HLA A2 transfectants. Staining 3G10 CTL with multimers of wild type and mutant HLA A2 presenting the YMDGTMSQV epitope confirmed that neither mutant HLA A2 was deficient in its ability to bind cell surface TCR (Fig. 4B).

We correlated these functional data to biochemical events within the CTL by investigating the differential effects present-

HLA A2 tetramers

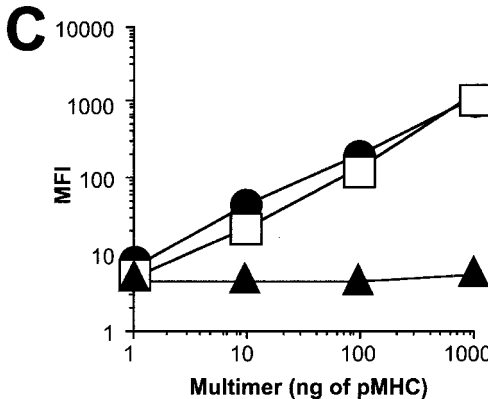
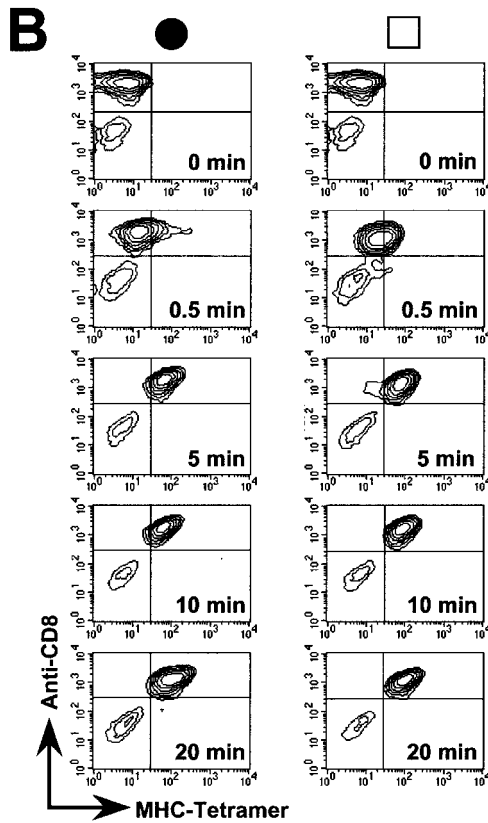
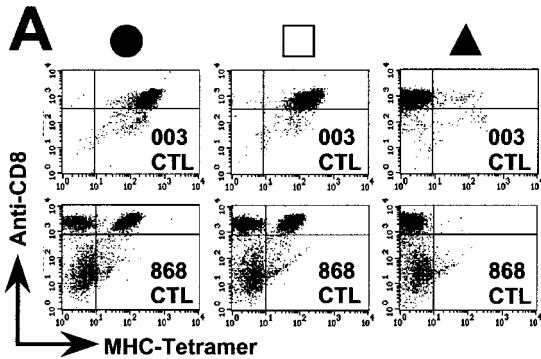
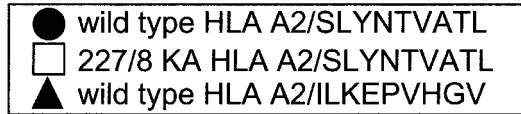


FIG. 2. Staining of 868 or 003 CTL with HLA A2 or D227K/T228A HLA A2 multimers. A, 868 or 003 CTL were stained in 20 μ l of PBS with 1 μ g of either SLYNTVATL-wild type HLA A2 (●) or SLYNTVATL-D227K/T228A HLA A2 (□) multimers (PE-conjugated) for 20

tation of antigen-pulsed C1R transfectants had on induction of the early TCR signal transduction cascade (Fig. 4C). We found that antigen recognition related most distinctly to the phosphorylation status of the CD3 ζ -chain, the phosphorylation of which represents the earliest biochemical event of the TCR signal transduction cascade. At high antigen concentration (10^{-5} M), both wild type HLA A2 and A245V HLA A2 transfectants, but not the D227K/T228A HLA A2 transfectant, induce the 23-kDa phosphoform of the CD3 ζ -chain. However, at lower antigen concentration (10^{-6} M), only wild type HLA A2 transfectants, but neither of the mutant HLA A2 transfectants, induces the 23-kDa phosphoform of the CD3 ζ -chain.

DISCUSSION

We investigated the contribution of the CD8 coreceptor to stabilization of the TCR/pMHC interaction and signal transduction during MHC class I-restricted activation of human CTL. Mutations in the $\alpha 3$ domain of MHC class I that fully abrogate binding to CD8 do not affect interaction with the TCR (Fig. 1). Despite this, cells expressing such mutant MHC class I do not activate CTL (Fig. 4). Multimers of a mutant MHC class I molecule (D227K/T228A; 227/8KA in Fig. 4) stain the CTL used in this study to a similar extent to wild type MHC class I multimers but fail to induce a normal TCR signal transduction cascade. It therefore appears that the CD8/pMHC interaction is not critical for the stable interaction of human pMHC multimers with cell surface TCR, but that this interaction is critical for physiological activation of human CTL. Furthermore, the CTL response to antigen was greatly reduced upon partial disruption of CD8 binding to pMHC by the A245V mutation (Fig. 4, 245V). These results clearly demonstrate that MHC class I-restricted T cell activation is not only dependent on this coreceptor but that the CD8/pMHC interaction enhances the sensitivity of CTL to antigen. Again we show that this enhanced sensitivity to antigen, mediated by CD8, is dependent on the ability of the coreceptor to effect signal transduction and does not derive from CD8-mediated stabilization of TCR-pMHC complexes.

We further investigated the biochemical basis for the observed coreceptor dependence of human CTL activation. The CD8/pMHC class I interaction is essential for induction of the 23-kDa phosphoform (p23) of CD3 ζ but is not required for induction of the 21-kDa phosphoform (p21) of CD3 ζ (Figs. 3A and 4C). Functional antigen recognition is characterized by the induction of both the p21 and p23 forms of CD3 ζ . The p23 form of CD3 ζ contains completely phosphorylated immunoreceptor tyrosine activation motifs and is thus critical for T cell activation, since it allows propagation of the TCR signal transduction cascade. In contrast, the p21 form of CD3 ζ contains only partially phosphorylated immunoreceptor tyrosine activation mo-

min at 37 °C. HLA A2 multimers presenting the irrelevant ILKEPVHGV peptide (▲) were used as control. Staining for CD8 with anti-CD8 monoclonal antibody (Tri-Color-conjugated; Caltag, Burlingame, CA) was carried out subsequent to the staining with pMHC multimer. B, 003 CTL were stained with anti-CD8-TriColor (Caltag) for 20 min at 37 °C. Cells were washed in RPMI 1640 and subsequently stained with either SLYNTVATL-wild type HLA A2 (●) or SLYNTVATL-D227K/T228A HLA A2 (□) multimers (PE-conjugated) at 37 °C for the indicated lengths of time. No multimer was added at the zero time point. Prestaining with anti-CD8-TriColor did not cause a significant change in the intensity of staining with pMHC multimer (data not shown) as has been observed with some antibodies in certain murine systems (39). C, 003 CTL stained with anti-CD8 (TriColor-conjugated) were washed and subsequently stained with indicated concentrations of SLYNTVATL-wild type HLA A2 (●), SLYNTVATL-D227K/T228A HLA A2 (□), or ILKEPVHGV-HLA A2 multimers (▲) (PE-conjugated) for 20 min at 37 °C. The graph plots mean fluorescence intensity (MFI) in the PE channel of the CD8-positive population against multimer concentration.

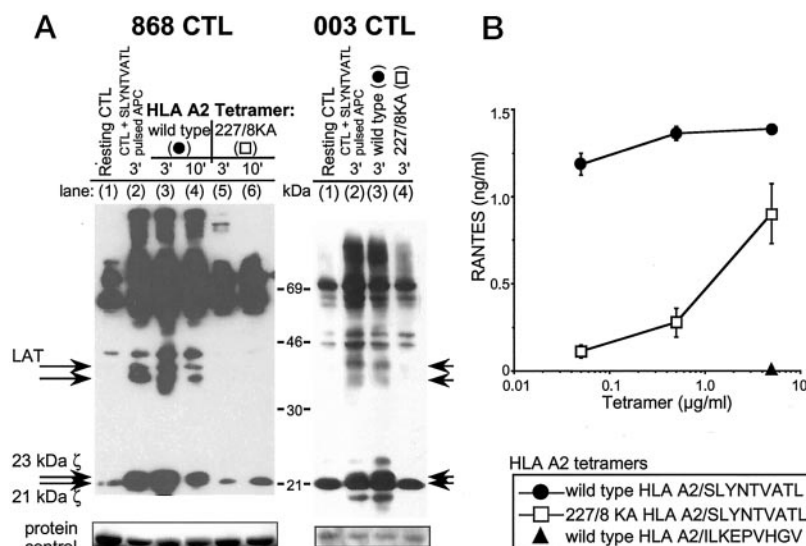


FIG. 3. Stimulation through the TCR by HLA A2 and D227K/T228A HLA A2 multimers. A, activation of early TCR signal transduction in response to wild type HLA A2 (●) or D227K/T228A HLA A2 (□) multimers. 003 and 868 CTL (10^6 /lane) were stimulated for the time indicated, and cell lysates were immunoblotted with anti-phosphotyrosine monoclonal antibody as described. *868 panel, lane 1*, resting CTL (no stimulus); *lane 2*, CTL stimulated for 3 min with autologous, Epstein-Barr virus-transformed B cells (10^6) pulsed with 800 nM SLYNTVATL peptide (an equivalent amount of peptide as carried on 50 μg/ml of pMHC); *lanes 3 and 4*, CTL stimulated with SLYNTVATL-wild type HLA A2 multimers (50 μg pMHC/ml) for either 3 min (*lane 3*) or 10 min (*lane 4*); *lanes 5 and 6*, CTL stimulated with SLYNTVATL-D227K/T228A HLA A2 multimers (50 μg of pMHC/ml) for either 3 min (*lane 5*) or 10 min (*lane 6*). The blot was reprobed with an anti-ZAP70 antibody to control for protein loading. *003 panel, lane 1*, resting CTL (no stimulus); *lane 2*, CTL stimulated with HLA-matched Epstein-Barr virus-transformed B cells (10^6) pulsed with 800 nM SLYNTVATL peptide; *lane 3*, CTL stimulated with SLYNTVATL-wild type HLA A2 multimer (50 μg of pMHC/ml); *lane 4*, CTL stimulated with SLYNTVATL-D227K/T228A HLA A2 multimer (50 μg of pMHC/ml). The blot was stained with Ponceau (Sigma) to control for protein loading. The staining of a prominent band is shown. To allow correlation between CTL activation and staining by HLA A2, the concentration of multimers used for staining (Fig. 2, *a* and *b*) and activation (Fig. 3*a*) is equivalent. B, release of RANTES by either 003 or 868 CTL (2×10^4 /well) in response to wild type HLA A2 (●) or D227K/T228A HLA A2 (□) multimers presenting the SLYNTVATL epitope was determined by enzyme-linked immunosorbent assay (R&D Systems). Extracellular RANTES concentration was determined 20 min after the addition of pMHC multimers. HLA A2 multimer presenting an irrelevant HIV-1-IIIB reverse transcriptase-derived (residues 476–484) peptide of sequence ILKEPVHGV was used as control (▲). Flow-through from MHC multimer solutions filtered through a 10-kDa cut-off membrane was added to control wells to demonstrate that activation was not due to free peptide in solution (data not shown).

tifs (29) and so cannot effect T cell activation. The p21 form of the ζ -chain may even inhibit T cell activation (30). Our observations thus indicate that the coreceptor dependence of antigen recognition by human CTL is afforded by the ability and extent of the CD8/pMHC class I interaction to mediate induction of the functionally active p23 form of CD3 ζ .

Our observations regarding the profile of ζ -chain phosphorylation in the presence and absence of the MHC/CD8 interaction may offer novel insights into the mechanism of TCR antagonism by altered peptide ligands. In both MHC class I and class II restricted systems of TCR antagonism, it has been demonstrated that antagonist altered peptide ligands induce the p21 form of CD3 ζ but, unlike agonist peptide, fail to effectively induce the p23 form of CD3 ζ (31–33). We establish here that preferential induction of the p21 phosphoform of the ζ -chain results from pMHC engagement of TCR in the absence of interaction with CD8. Thus, TCR engagement of an agonist ligand in the absence of a CD8/pMHC interaction leads to the induction of an early protein tyrosine phosphorylation profile similar to that induced by antagonist ligands. It has been postulated that pMHC engagement with TCR precedes engagement with coreceptor, thus making coreceptor engagement dependent on the stability of the TCR-pMHC complex (34). The short half-lives of TCR-antagonist pMHC complexes (35) could preclude sufficient recruitment of CD8/p56^{lck} to the TCR-induced signaling complex. The incomplete participation of CD8/p56^{lck} in the transmembrane complex could then lead to partial phosphorylation of the CD3 ζ -chain and abortive CTL activation.

The finding, using specific point mutations in human MHC class I, that CD8 is not obligatory for stabilizing the interaction between TCR and MHC is in agreement with data indicating

that CD4 plays no role in forming a stable TCR interaction with multimeric pMHC class II ligands (36–38). However, our findings contrast with antibody-derived conclusions from two murine systems investigating H2-K^b-restricted TCRs (2C and OVA), which suggest that the murine CD8/pMHC interaction accounts for 85–100% of the stability of murine pMHC multimers bound to cell surface 2C or OVA TCR, respectively (39). Thus, it appears that the cell surface binding of mouse pMHC multimers could be more dependent on the CD8/pMHC interaction than for human multimers. Interestingly, while binding studies on the murine CD8/pMHC interaction are consistent with a cooperative role for CD8 in stabilizing the interaction of TCR with MHC (9), our analogous binding data using human proteins tends to exclude this notion (11). Recent data from another laboratory (40) suggest that the use of multimers of pMHC class I with reduced CD8 binding can cause some reduction in the intensity of staining of some CTL clones. We too have observed up to a maximal 50% reduction in the staining of some CTL with D227K/T228A-mutated MHC class I multimers (41). These data indicate that CD8 can contribute to a limited degree to the stabilization of some cell surface human TCR/pMHC interactions. This contribution appears to be less than that documented in murine systems (39). Consequently, there may be a spectrum for CD8-dependent stabilization of the TCR/pMHC interaction. Results to date indicate that human and murine TCRs may fall at opposite ends of this spectrum. The differences in the affinity of the CD8/pMHC interaction between murine and human systems (K_D values of 30–86 and 130–220 nM, respectively; reviewed in Ref. 1) also appear consistent with this concept. However, it remains disputed whether the different dissociation constants determined for the published murine and human CD8/pMHC interaction repre-

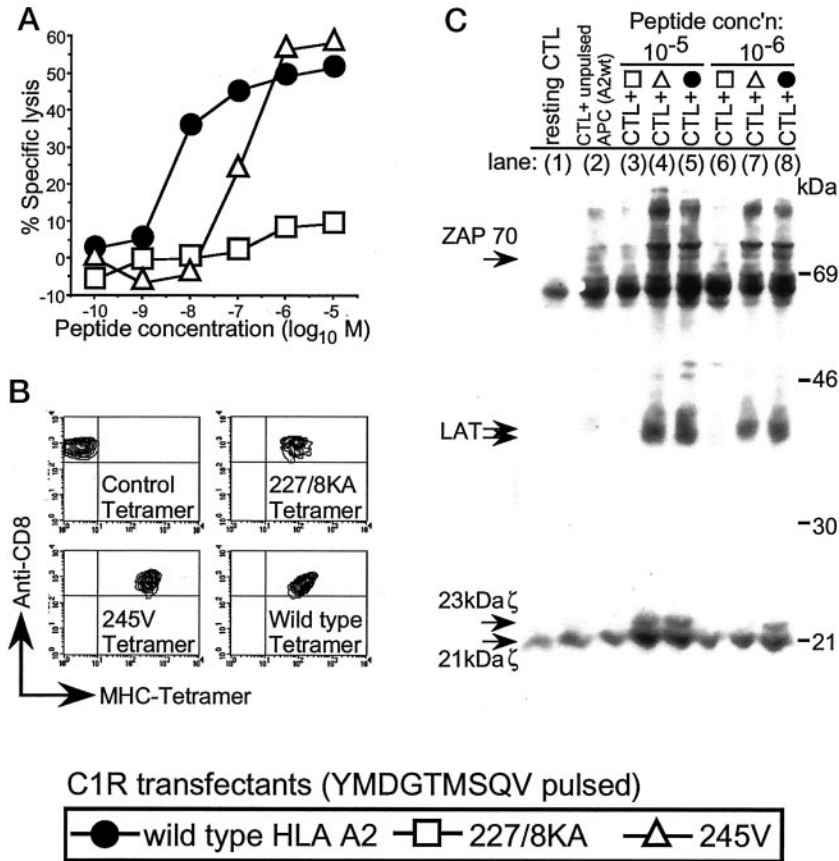


FIG. 4. CD8 is essential for CTL-mediated cytotoxicity and sensitivity to antigen. *A*, lysis of antigen-pulsed C1R cells stably transfected to express wild type HLA A2 (●), D227K/T228A HLA A2 (□), or A245V HLA A2 (△). C1R cells were stably transfected to express wild type MHC, D227K/T228A HLA A2, or A245V HLA A2. C1R transfectants pulsed with the HLA A2-restricted melanoma-derived epitope YMDGTMSQV were used as targets for melanoma-specific CTL clone 3G10 (13) in a 4-h chromium release assay. *B*, 3G10 CTL were stained in 20 μl of PBS with 4 μg of wild type HLA A2, D227K/T228A HLA A2, or A245V HLA A2 multimers presenting the YMDGTMSQV epitope as described. Staining for CD8 with anti-CD8 was carried out subsequent to the staining with MHC multimer. Multimers of wild type HLA A2 presenting the SLYNTVATL epitope were used as a negative control. *C*, induction of early signal transduction by HLA A2 transfectant C1R cells. C1R transfectants were pulsed with the indicated concentration of index (YMDGTMSQV) peptide. Peptide-pulsed C1R transfectants (10⁵) were presented to 3G10 CTL (10⁶) for 10 min. Cells were subsequently lysed, and cell lysates were immunoblotted with anti-phosphotyrosine monoclonal antibody. *Lane 1*, resting CTL (no APC). *Lane 2*, CTL exposed to unpulsed C1R expressing wild type HLA A2. *Lane 3*, CTL exposed to D227K/T228A HLA A2-expressing C1R cells pulsed with 10 μM index peptide. *Lane 4*, CTL exposed to A245V HLA A2-expressing C1R cells pulsed with 10 μM index peptide. *Lane 5*, CTL exposed to wild type HLA A2-expressing C1R cells pulsed with 10 μM index peptide. *Lanes 6–8*, as in *lanes 3–5*, respectively, except that transfectants were pulsed with 1 μM index peptide.

TABLE I
Affinities of mouse and human CD8 for mouse and human pMHC complexes

Biotinylated pMHC complexes H-2K^b and HLA A2 were immobilized on a streptavidin-coated BIAcore chip, and varying concentrations of mouse or human soluble CD8α were flowed over the chip surface at 25 °C. Binding of human CD8 to mouse pMHC is the same as the binding of mouse CD8 to mouse pMHC. However, mouse CD8 does not bind detectably to human pMHC, but binding of human CD8 to human pMHC is similar to that determined previously (11). In order to maximize the reliability of this comparison, these experiments were performed directly after one another on the same BIAcore chip. On a second chip, the binding of human and mouse CD8 to mouse pMHC made with mouse β2m was compared with mouse pMHC made with human β2m (as is commonly used for making pMHC tetramers). There is a slightly higher affinity of human CD8 for mouse pMHC made with human β2m (*K_D* is 38% lower), but there is less significant difference (*K_D* is 15% higher) in the case of mouse CD8.

Chip	Immobilized complex	<i>K_D</i>	
		Human CD8 binding	Mouse CD8 binding
<i>μM</i>			
1	Mouse H-2K ^b /mouse β2m	30 ± 0.8	31 ± 0.8
1	Human HLA A2/human β2m	149 ± 12	No binding
2	Mouse H-2K ^b /human β2m	18 ± 0.5	31 ± 1.4
2	Mouse H-2K ^b /mouse β2m	29 ± 0.6	27 ± 1.0

sent a true difference in affinity or simply reflect disparate experimental conditions. To address this issue, we measured the interaction of murine and human sCD8α with murine and human pMHC class I (H-2K^b and HLA A2) by SPR (Table I). In order to maximize the reliability of this comparison, these experiments were performed directly after one another on the same BIAcore chip. These results confirm previous findings and indicate that the murine CD8/pMHC interaction is signif-

icantly stronger than the human CD8/pMHC interaction. This finding is consistent with differences in the human and murine CD8-pMHC cocrystal structures (10, 42). While the overall topology of the human CD8α-HLA A2 complex and murine CD8α-H-2K^b complex are similar, there is considerably less contact between the interacting molecules in the human *versus* the mouse (1821 Å² compared with 2554 and 2591 Å²) (42).

Human CD8 binds better to murine MHC than to human

MHC (Table I). In contrast, we were unable to detect binding of murine CD8 to human MHC. Human $\beta 2m$ is commonly used in the manufacture of murine pMHC multimers. The inclusion of human $\beta 2m$ in the murine pMHC complex does not appear to significantly alter the CD8/pMHC interaction (Table I).

Equilibrium binding-derived K_D values for agonist TCR/pMHC interactions are reported to vary from below 1 μM for a human HLA A2 Tax-specific TCR (43) to over 50 μM for the 3.L2 murine TCR (44). If such variation exists, it is clear that the ratio of the contribution made by the TCR and CD8 to stabilization of pMHC multimers to the cell surface can vary widely. It is tempting to speculate that a stronger TCR/pMHC interaction results in CD8-independent staining with pMHC multimers. It is also possible that the degree of dependence on the co-receptor for T cell activation correlates with the affinity of individual TCR/pMHC interactions, although further work is required to test this hypothesis.

We further note that the rapid activation of CTL by pMHC multimers may result in an undesirable perturbation of intracellular processes when MHC multimers are used as diagnostic tools, for example in the phenotyping of antigen-specific CD8⁺ lymphocytes. Mutant MHC class I multimers that specifically stain but do not activate CTL may prove useful in circumventing activation-related complications. Indeed, a recent study using the A245V mutation (independently also used in this study) indicated that multimers of this molecule might exhibit greater specificity for antigen-specific CTL (40). Similar or enhanced findings may apply to multimeric forms of MHC class I with a D227K/T228A substitution, which completely abrogates the CD8/pMHC interaction (Fig. 1). Thus, the use of D227K/T228A multimers may exhibit the dual benefit of increased specificity and less intracellular perturbation. However, since antigenic stimulation of CTL in the absence of the CD8/pMHC interaction can lead to activation induced cell death of CTL (41, 45) some functional signaling might be induced by the D227K/T228A mutated reagents used in this study.

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