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MHC Class I Molecules with Superenhanced CD8 Binding Properties Bypass the Requirement for Cognate TCR Recognition and Nonspecifically Activate CTLs

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CD8$^+$ CTLs are essential for effective immune defense against intracellular microbes and neoplasia. CTLs recognize short peptide fragments presented in association with MHC class I (MHCI) molecules on the surface of infected or dysregulated cells. Ag recognition involves the binding of both TCR and CD8 coreceptor to a single ligand (peptide MHCI [pMHCI]). The TCR/pMHCI interaction confers Ag specificity, whereas the pMHCI/CD8 interaction mediates enhanced sensitivity to Ag. Striking biophysical differences exist between the TCR/pMHCI and pMHCI/CD8 interactions; indeed, the pMHCI/CD8 interaction can be >100-fold weaker than the cognate TCR/pMHCI interaction. In this study, we show that increasing the strength of the pMHCI/CD8 interaction by ~15-fold results in nonspecific, cognate Ag-independent pMHCI tetramer binding at the cell surface. Furthermore, pMHCI molecules with superenhanced affinity for CD8 activate CTLs in the absence of a specific TCR/pMHCI interaction to elicit a full range of effector functions, including cytokine/chemokine release, degranulation and proliferation. Thus, the low solution binding affinity of the pMHCI/CD8 interaction is essential for the maintenance of CTL Ag specificity. ***The Journal of Immunology*****, 2010, 184: 3357–3366.**

CD8$^+$ CTLs recognize antigenic determinants in the form of short peptides derived from endogenous proteins bound to MHC class I (MHCI) molecules on the surface of target cells and play a critical role in immune defense against intracellular pathogens and tumors. Ag specificity is conferred by the TCR, which interacts with the peptide-binding platform formed by the α1 and α2 domains of MHCI (1, 2). In contrast, the surface gp CD8 binds to invariant regions of MHCI and is capable of enhancing cellular sensitivity to Ag by up to six orders of magnitude (3, 4). CD8 mediates this profound enhancement of Ag sensitivity through a number of distinct mechanisms: 1) enhancement of the TCR/peptide MHCI (pMHCI) association rate (5–7); 2) stabilization of the TCR/pMHCI interaction (8, 9); 3) recruitment of essential kinases to the intracellular side of the TCR/CD3/ζ complex (10, 11); and 4) localization of TCR/pMHCI complexes within specialized membrane microdomains that are enriched for early intracellular signal transduction molecules and are thought to act as privileged sites for TCR-mediated cascade initiation (12, 13).

The MHCI binding site for CD8 is separate from the peptide-binding domains that are recognized by the TCR (2) and this spatial segregation allows both TCR and CD8 to bind a single MHCI molecule simultaneously (14). Thus, CTL recognition of Ag involves the binding of two receptors (TCR and CD8) to a single ligand (pMHCI), a modus operandi that is unique to αβ T cell biology. The pMHCI/CD8 interaction is characterized by very low solution affinities (K_D ~150 μM) and rapid kinetics (K_{off} ~18 s^{-1}) (15, 16). Indeed, the affinity of the pMHCI/CD8 interaction is even lower than the corresponding values measured for conventional molecular binding events involved in cell–cell recognition, such as the CD2/CD48 interaction (K_D = 60–90 μM) (15, 17). In stark contrast, the TCR/pMHCI interaction can be more than 100-fold stronger than the pMHCI/CD8 interaction (K_D range for agonists from 0.14 μM, the strongest natural TCR/pMHCI interaction measured to date) and exhibits considerably slower kinetics (K_{off} range for agonists 0.01–1 s^{-1}) (1, 6, 18–20). It seems extremely unlikely that the striking biophysical characteristics of the pMHCI/CD8 interaction have occurred by accident. Indeed, this conclusion is strengthened by the finding that the pMHCI/CD8 interaction is capable of exerting the vast majority of its biological function when weakened even further (21), which suggests that CD8 has specifically evolved to operate at very low solution affinities.

In this study, we probe the functional significance of the low solution affinity pMHCI/CD8 interaction using pMHCI molecules with superenhanced CD8 binding properties. Notably, we find that pMHCI molecules with affinities for CD8 that lie within the typical range for agonist TCR/pMHCI interactions (K_D ~10 μM) are able to activate CTL in the absence of a specific TCR/pMHCI interaction. Thus, the biophysical

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Abbreviations used in this paper: 7-AAD, 7-amino-actinomycin D; C1R, Hmy.2 C1R B; hTERT, human telomerase reverse transcriptase; MHCI, MHC class I; pMHCI, peptide MHCI.

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characteristics of the pMHCI/CD8 interaction are essential for the maintenance of CTL Ag specificity.

Materials and Methods

Cells

The CTL clones 003 and NT1 and the CTL line 868 are all specific for the HIV-1 p17 Gag-derived epitope SLYNTVATL (residues 77–85) restricted by HLA A*0201 (A2 from this point forward) (22, 23). The following A2-restricted CTL clones were also used in this study: 1) Mel13, specific for the Melan-A–derived epitope ELAGIGILTV (residues 26–35); and 2) ILA1, specific for the human telomerase reverse transcriptase (hTERT)-derived epitope ILAKFLHWL (residues 540–548) (6, 24). In addition, the following non-A2–restricted CTL clones were used: 1) the HLA A*6801-restricted CTL clone c23, specific for the HIV-1 Tat-derived epitope ITKGLGISYGR (residues 38–48) (25); 2) the HLA B*0702-restricted CTL clone KD4, specific for the EBV EBNA3A-derived epitope RPPIFIRRL (residues 379–387); 3) the HLA B*0801-restricted CTL clone LC13, specific for the EBV EBNA3A-derived epitope FLRGRAYGL (residues 339–347) (26, 27); and 4) the HLA B*3508-restricted CTL clone SB27, specific for the EBV BZLF1-derived epitope LPEPLPQGQLTAY (residues 52–64) (28, 29). All CTLs were maintained in RPMI 1640 (Life Technologies, Rockville, MD) containing 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), 2 mM L-glutamine (Life Technologies), and 10% heat inactivated FCS (Life Technologies) (R10) supplemented with 2.5% Cellkines (Helvetica Healthcare, Geneva, Switzerland), 200 IU/ml IL-2 (PeproTech, Rocky Hill, NJ) and 25 ng/ml IL-15 (PeproTech). PBMCs were isolated by standard Ficoll-Hypaque density gradient centrifugation from healthy donor blood. The 293T-CD8α cells were manufactured by introducing pBullet-human CD8α (30, 31) into 293T cells using vesicular stomatitis virus-pseudotyped Moloney murine leukemia virus particles. The 293T-CD8α cells were cultured in DMEM (Life Technologies) supplemented with 20% FCS (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), and 2 mM L-glutamine (Life Technologies). Hmy.2 C1R B (C1R) cells expressing full-length A2 and variants thereof were generated as described previously (21).

pMHCI tetramers

Tetrameric complexes of wild-type pMHCI molecules and mutants thereof were produced, stored and used as described previously (9, 21). The following A2-restricted peptide epitopes were used to refold the pMHCI molecules used in this study: SLYNTVATL (HIV-1 p17 Gag, residues 77–85), LLFGYPVYV (HTLV-1 Tax, residues 11–19), GLCTLVAML (EBV BMLF1, residues 259–267), NLVPMVATV (CMV pp65, residues 495–503), ELAGIGILTV (Melan-A, residues 26–35), and ILAKFLHWL (hTERT, residues 540–548). Tetrameric or multimeric pMHCI reagents were constructed by the addition of streptavidin conjugated to PE, quantum dot 605 or quantum dot 800 (Life Technologies) at the appropriate molar ratios.

Abs

The following mAbs were used in this study: purified anti-human CD8 (clone DK-25; Dako, Carpinteria, CA), allophycocyanin-conjugated anti-human CD8 (clone RPA-T8; BD Biosciences, San Jose, CA), FITC-conjugated anti-human CD8 (clone SK1; BD Biosciences), PerCP-conjugated anti-human CD8 (clone SK1; BD Biosciences), PE-conjugated anti-human CD8β (clone 2ST8.5H7; Beckman Coulter, Fullerton, CA), PerCP-conjugated anti-human CD3 (clone SK7; BD Biosciences), FITC-conjugated anti-human αβ-TCR (clone BMA 031; Serotec, Oxford, U.K.), FITC-conjugated or PE-conjugated anti-human γδ-TCR (clone YB5.B8; BD Pharmingen, San Diego, CA), allophycocyanin-conjugated anti-human CD56 (clone AF12-7H3; Miltenyi Biotec, Auburn, CA), FITC-conjugated anti-human CD56 (clone MEM188; Caltag Laboratories, Burlingame, CA), FITC-conjugated anti-A2 (clone BB7.2; Serotec), and FITC-conjugated anti-human CD107a (clone H4A3; BD Biosciences). Unless specified, the anti-human CD8 mAbs used in this study target the α-chain of the coreceptor dimer. Dead cells were excluded from flow cytometric analyses with 7-amino-actinomycin D (7-AAD; BD Biosciences).

Flow cytometry

For pMHCI tetramer staining, 2.5×10^6 PBMC, 5×10^4 CTLs or 2×10^5 293T cells (untransfected or CD8α-transfected) were resuspended in PBS or FACS buffer (2% FCS/PBS) and stained with pMHCI tetramer at the concentrations indicated for 20–30 min at 37°C. Cells were subsequently stained with combinations of the mAbs described previously for 30 min on ice. Prior to staining, 293T cells were treated with Versene (Life Technologies) for 10 min at 37°C. For anti-CD8 mAb blocking experiments: 2.5×10^6 PBMCs were pretreated with 10 μg/ml unconjugated anti-CD8 mAb (clone DK-25; Dako) for 20 min on ice prior to staining with 10 μg/ml pMHCI tetramer for 45 min on ice. For A2 typing: 2.5×10^6 PBMCs were stained with 5 μl FITC-conjugated anti-A2 mAb (clone BB7.2; Serotec) for 30 min on ice. Samples were then washed twice and resuspended in PBS. Data were acquired using a FACSCalibur or FACSAria II flow cytometer (BD Biosciences) and analyzed with either CellQuest (BD Biosciences) or FlowJo (Tree Star, Ashland, OR) software.

TCR downregulation assay

The 10^5 003 CTLs per well were resuspended in a 96-well round-bottomed plate with various concentrations of the indicated PE-conjugated tetramers (A2 SLYNTVATL, A2/K^b SLYNTVATL, A2 LLFGYPVYV, or A2/K^b LLFGYPVYV) diluted in 40 μl RPMI 1640 containing 2% FCS plus penicillin, streptomycin, and glutamine as described previously (R2) for 30 min at 37°C. Cells were then washed, resuspended in ice-cold azide buffer (0.1% azide/2% FCS/PBS), and subsequently stained with FITC-conjugated anti–αβ-TCR (clone BMA 031; Serotec), 7-AAD (BD Biosciences), and allophycocyanin-conjugated anti-CD8 (clone RPA-T8; BD Biosciences) for 30 min on ice. After two additional washes, cells were resuspended in ice-cold azide buffer. Data were acquired using a FACSCalibur flow cytometer and analyzed with CellQuest software (BD Biosciences).

Cytokine/chemokine assays: ELISA, cytometric bead array, and ELISPOT

CTLs were incubated with C1R A2 cells, C1R A2/K^b cells, or medium alone at different E:T ratios overnight at 37°C. Subsequent to incubation, the supernatant was harvested and assayed for MIP-1β, IFN-γ, or RANTES by ELISA (R&D Systems, Minneapolis, MN). Remaining supernatant was assayed with the human Th1/Th2 cytokine kit (BD Biosciences) according to the manufacturer's instructions; data were acquired using a FACSCalibur flow cytometer and analyzed with CBA software (BD Biosciences). For tetramer-based ELISPOT assays, 2×10^3 CTL ± pMHCI tetramer at 1 μg/ml were applied to duplicate wells of PVDF-backed plates (Millipore, Bedford, MA) precoated with IFN-γ capture Ab 1-DIK (Mabtech, Nacka, Sweden) in a total volume of 200 μl R2 and incubated for 4 h at 37°C. To exclude activation by cognate peptide representation or fluorochrome-mediated aggregation, cognate A2 D227K/T228A tetramers were included as controls; these tetramers do not bind CD8 and did not activate 003 or 868 CTLs, despite efficient staining in both cases (data not shown). Plates were developed according to the manufacturer's instructions (Mabtech) and spots were counted using an automated ELISpot Reader System ELR02 (Autoimmun Diagnostika GmbH, Strassberg, Germany).

Degranulation assay

Surface CD107a mobilization was used to assess degranulation as described previously (32). Briefly, CTLs were incubated for 4 h at 37°C with either C1R A2 cells, C1R A2/K^b cells or medium alone at different E:T ratios; alternatively, CTLs were incubated with various pMHCI tetramers. Both FITC-conjugated anti-CD107a (clone H4A3; BD Biosciences) and 0.7 μl/ml monensin (GolgiStop; BD Biosciences) were added prior to incubation. Subsequent to incubation, the cells were washed twice and resuspended in PBS. Data were acquired using a FACSCalibur flow cytometer and analyzed with FlowJo software (Tree Star).

CTL priming assay

Transfected C1R cells were pulsed with 1 μM ELAGIGILTV (Melan-$A_{26–35}$) peptide for 90 min, irradiated, and washed once in RPMI 1640 medium. Pulsed, irradiated C1R cells (2×10^5) were incubated with 10^6 fresh $A2^+$ human PBMCs in R10; 200 IU/ml IL-2 was added on day 3. $CD8^+$ cells specific for Melan-$A_{26–35}$ were quantified on day 10 with wild-type A2 ELAGIGILTV tetramer.

Results

Generation of MHCI molecules with superenhanced CD8 binding affinity

Tetrameric fusion molecules comprising the α1/α2 peptide binding platform of A2 and the α3 domain of H2-K^b (A2/K^b from this point forward) enable the monitoring of $CD8^+$ T cell responses in A2 transgenic mice (33). This reflects a requirement for the murine MHCI α3 domain to engage murine CD8 (11), thus enabling A2/K^b reagents to stain murine CTL with lower affinity TCR/pMHCI interactions (so-called "low avidity" CTLs) (22). The A2/K^b H chain folded with human β2m interacts strongly with human CD8 (K_D ~10 μM, compared with A2 that binds to CD8 with a K_D ~150 μM) but exhibits unaltered A2-restricted TCR binding properties (9, 22). Thus, fusing the murine α3 domain with A2 α1/α2 domains increases

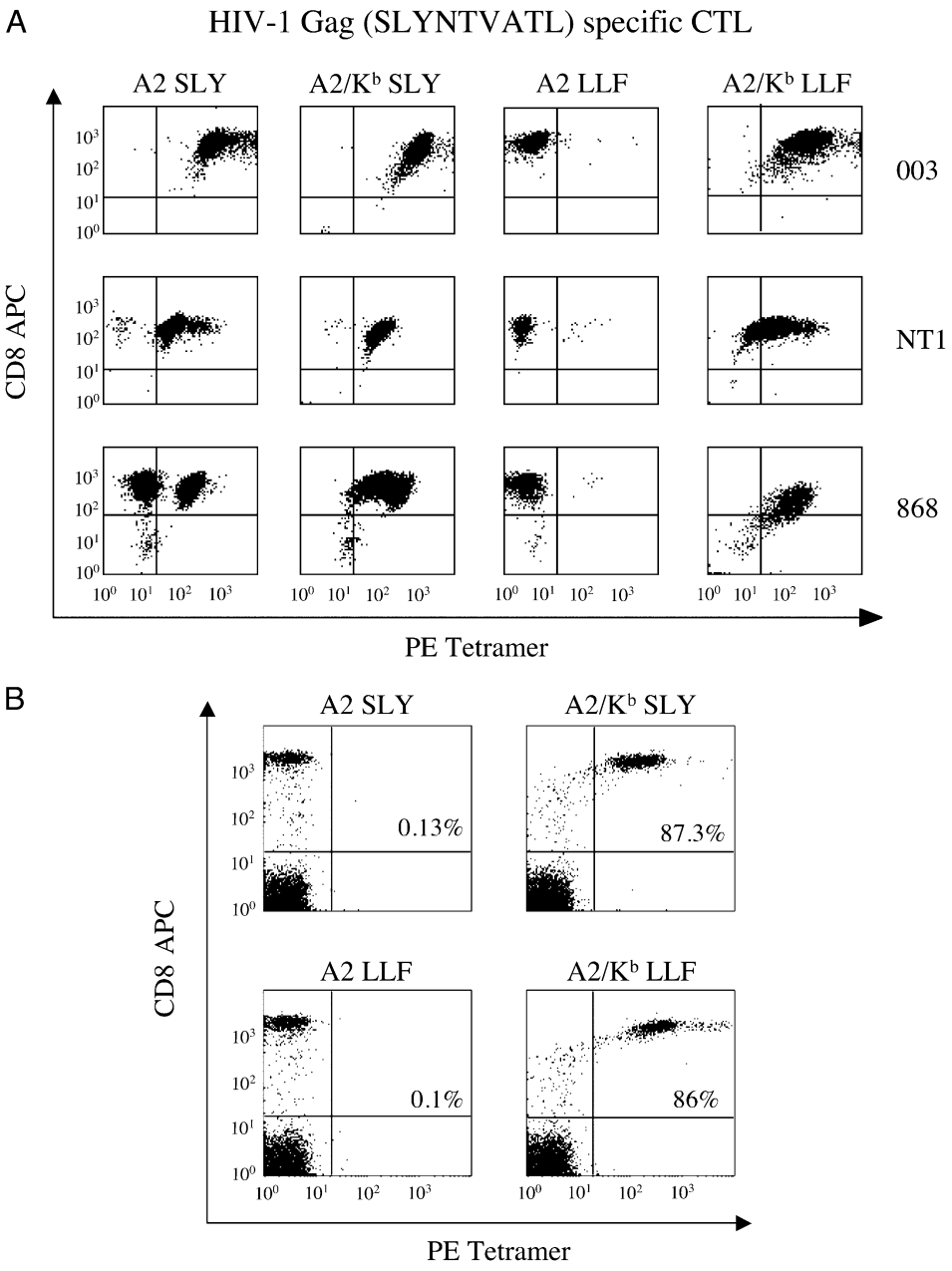

FIGURE 1. The exquisite specificity of pMHCI tetramer staining is lost when the strength of the pMHCI/CD8 interaction is increased by ~15-fold. *A*, The 003 or NT1 CTL clones (10^5 cells) or the 868 CTL line (2.5×10^5 cells), all specific for HIV-1 p17 $Gag_{77–85}$, were stained with 1 μg of the PE-conjugated tetramers A2 SLYNTVATL, A2/K^b SLYNTVATL, A2 LLFGYPVYV, or A2/K^b LLFGYPVYV in 20 μl PBS for 20 min at 37°C. Cells were then stained with allophycocyanin-conjugated anti-CD8 and 7-AAD for 30 min on ice, washed twice, and resuspended in PBS. Data were acquired using a FACSCalibur flow cytometer and analyzed with CellQuest software. *B*, 2.5×10^5 PBMCs were suspended in 250 μl FACS buffer (2% FCS/PBS), then stained with 1 μg of the PE-conjugated tetramers A2 SLYNTVATL, A2/K^b SLYNTVATL, A2 LLFGYPVYV, or A2/K^b LLFGYPVYV for 20 min at 37°C. Each sample was subsequently stained with allophycocyanin-conjugated anti-CD8, PerCP-conjugated anti-CD3, and 7-AAD for 30 min on ice, washed twice, and resuspended in FACS buffer. Data were acquired using a FACSCalibur flow cytometer and analyzed with CellQuest software by gating on the live $CD3^+$ population. The values shown represent the percent of $CD3^+$ $CD8^+$ cells that stain with the indicated tetramer.

the strength of the pMHCI/CD8 interaction by ~15-fold without affecting the TCR/pMHCI interaction.

Superenhanced CD8 binding results in nonspecific pMHCI ligand interactions

Monomeric pMHCI complexes cannot be used to examine TCR/pMHCI binding at the cell surface because of the extremely short half-life of such interactions. Increasing the valency of these molecules by avidin/biotin-based tetramerization overcomes this limitation and produces reagents that are invaluable for the identification and characterization of Ag-specific CTLs (34, 35). Indeed, it is well established that wild-type tetrameric pMHCI reagents bind to cell surface TCR with exquisite specificity (34, 36). Thus, A2/K^b tetrameric reagents were generated to study the effect of superenhanced CD8 binding on the specificity of pMHCI ligand interactions at the cell surface.

Wild-type pMHCI tetrameric reagents bearing cognate peptide stained three distinct A2-restricted CTLs specific for SLYNTVATL (HIV-1 p17 $Gag_{77–85}$), each expressing a different TCR (19, 22, 23) (Fig. 1*A*). Noncognate A2 LLFGYPVYV (HILV-1 $Tax_{11–19}$) tetramers failed to stain any of these in vitro expanded CTL populations to any notable extent. However, A2/K^b LLFGYPVYV tetramers stained all SLYNTVATL-specific CTLs; in addition, both the A2/K^b SLYNTVATL and A2/K^b LLFGYPVYV tetramers stained the noncognate $CD8^+$ cell population in the 868 CTL line (Fig. 1*A*). To examine this effect in more detail, we used A2 and A2/K^b tetramers to stain fresh human PBMCs. Ag-specific $CD8^+$ cell populations were not identified in PBMCs from healthy donors with either the A2 SLYNTVATL or A2 LLFGYPVYV tetramers (Fig. 1*B*). In contrast, both the A2/K^b SLYNTVATL and A2/K^b LLFGYPVYV tetramers stained >85% of $CD8^+$ cells in PBMCs (Fig. 1*B*); similar data were obtained with A2/K^b GLCTLVAML (EBV $BMLF1_{259–267}$) and A2/K^b NLVPMVATV (CMV $pp65_{495–503}$) tetramers (data not shown). Taken together, these data indicate that the exquisite specificity of tetrameric pMHCI reagents is lost when the strength of the pMHCI/CD8

interaction is increased by ~15-fold. Thus, the low solution affinities of the wild-type pMHCI/CD8 interaction are required to maintain pMHCI binding specificity at the cell surface.

A2/K^b tetramers bind the majority of CTLs in peripheral blood

Noncognate A2/K^b tetramers were observed to bind ~80% of the CD8α$^+$ population in peripheral blood (Fig.1*B*). Although CD8α is predominantly found on the surface of αβ-TCR$^+$ CTLs, it is also found on the surface of other lymphocytes, most notably some γδ T cells and NK cells. We therefore sought to determine the identity of the CD8α$^+$ cells that stain with A2/K^b tetramers. Staining of fresh ex vivo PBMCs isolated from healthy A2$^+$ donors revealed that CD8α was expressed on ~39%, 54%, and 32% of the αβ-TCR$^+$, NK cells, and γδ-TCR$^+$ populations, respectively, with some variation between donors (Fig. 2*A*). The majority of γδ-TCR$^+$ (~93.6%) and NK cells (~77%) failed to stain with the A2/K^b ILAKFLHWL (hTERT$_{540–548}$) tetramer and no significant binding was observed with the corresponding A2 tetramer (Fig. 2*B*). However, the vast majority of αβ-TCR$^+$/CD8$^+$ cells within the lymphocyte population stained nonspecifically with the A2/K^b ILAKFLHWL tetramer (Fig. 2*C*).

We hypothesized that most γδ-TCR$^+$ cells and NK cells might fail to bind A2/K^b tetramers because they express the CD8αα homodimer rather than the CD8αβ heterodimer, which is expressed on the surface of CTLs. Thus, we generated a 293T cell line that expressed CD8αα (Fig. 3*A*) to examine the ability of A2/K^b tetramers to bind this homodimeric form of the CD8 coreceptor on the cell surface. In contrast to both A2 and A2 D227K/T228A tetramers, which exhibit normal and abrogated interactions with CD8, respectively, A2/K^b tetramers bound to most (74.3%) of the CD8αα$^+$ 293 T cell transfectants (Fig. 3*A*, 3*B*); no binding was observed in the absence of CD8αα surface expression (Fig. 3*A*). Thus, A2/K^b tetramers are capable of binding to cell surface CD8αα.

Why do A2/K^b tetramers bind predominantly to the CTL population in peripheral blood and not to other cells that express CD8? Fig. 3*B* shows that A2/K^b tetramer staining is directly proportional to the level of CD8αα expression, such that only cells with a higher level

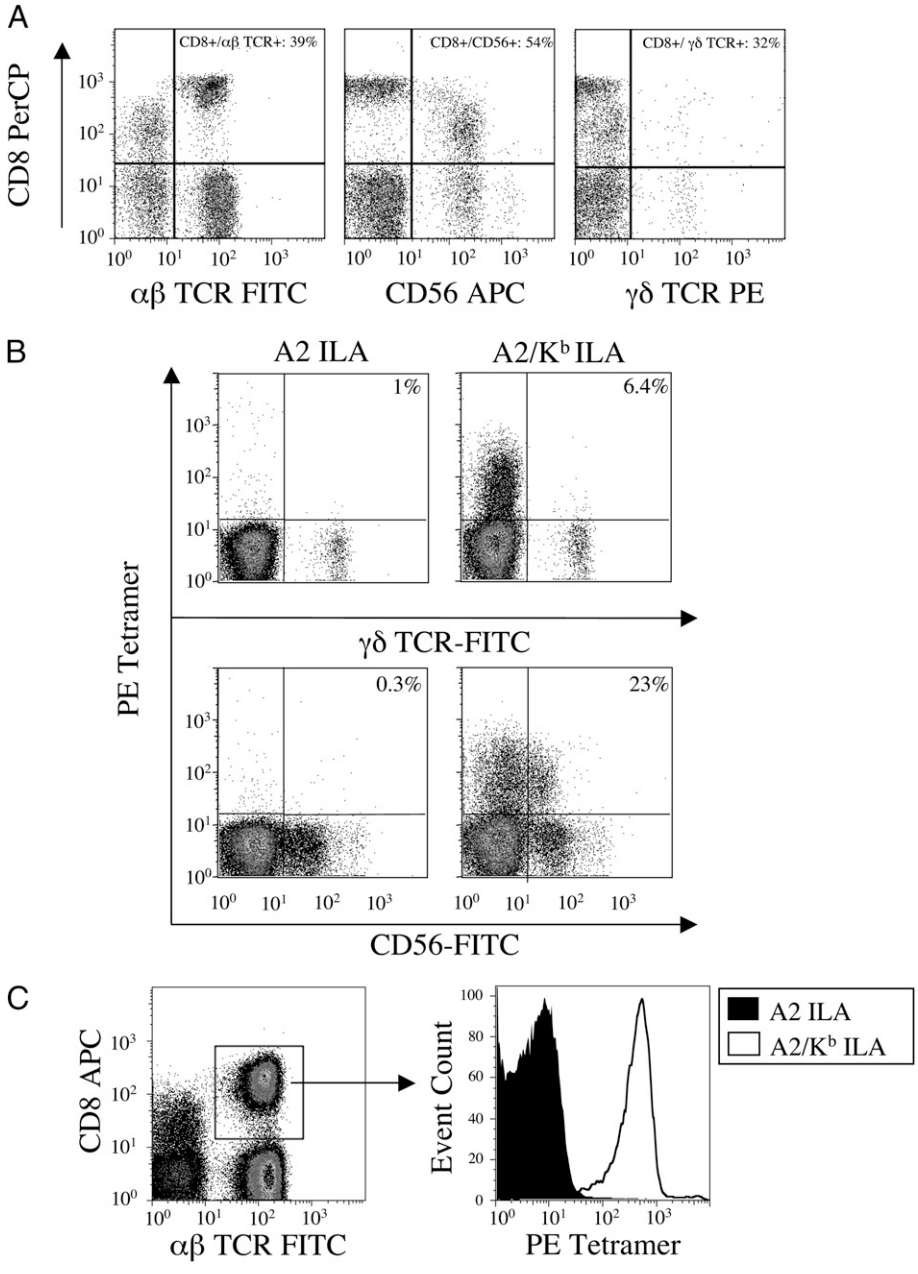

FIGURE 2. A2/K^b tetramers bind the majority of CTLs in peripheral blood. *A*, 2.5 × 10^5 PBMCs from an A2$^+$ donor were stained with PerCP-conjugated anti-CD8, 7-AAD, and either FITC-conjugated anti–αβ-TCR, allophycocyanin-conjugated anti-CD56 or PE-conjugated anti–γδ-TCR for 30 min on ice, washed twice, and resuspended in PBS. *B*, 2.5 × 10^5 A2$^+$ PBMCs were stained with 10 μg/ml of the PE-conjugated tetramers A2 ILAKFLHWL or A2/K^b ILAKFLHWL for 20 min at 37°C. After washing, cells were subsequently stained with 7-AAD and either FITC-conjugated anti–γδ-TCR or FITC-conjugated anti-CD56 for 30 min on ice, washed twice, and resuspended in PBS. *C*, 2.5 × 10^5 A2$^+$ PBMCs were stained with 10 μg/ml of the PE-conjugated tetramers A2 ILAKFLHWL or A2/K^b ILAKFLHWL for 20 min at 37°C. After washing, cells were stained with allophycocyanin-conjugated anti-CD8, FITC-conjugated anti–αβ-TCR and 7-AAD for 30 min on ice, washed twice, and resuspended in PBS. In *A*, *B*, and *C*, data were acquired using a FACSCalibur flow cytometer and analyzed with FlowJo software.

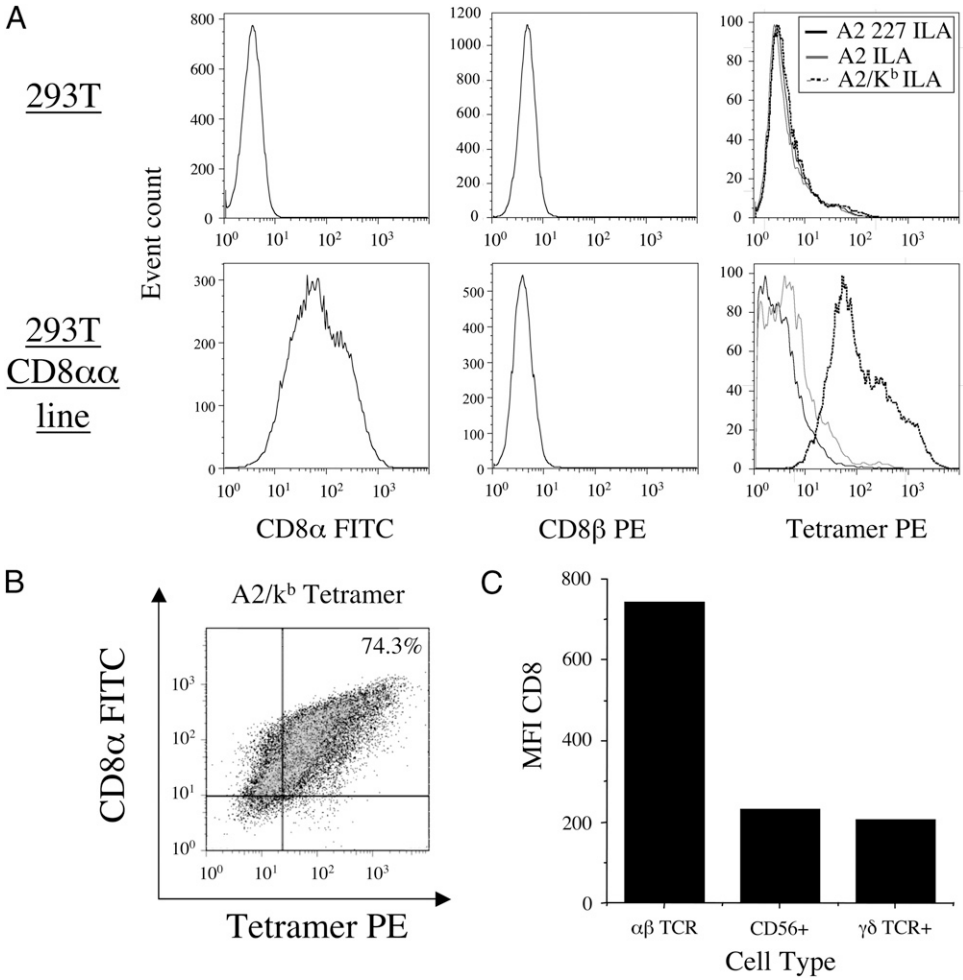

FIGURE 3. Nonspecific A2/K^b tetramer binding is influenced by CD8 cell surface density. *A* and *B*, 2×10^5 293T cells were incubated ± 10 μg/ml of the PE-conjugated tetramers A2 D227K/T228A ILAKFLHWL, A2 ILAKFLHWL, or A2/K^b ILAKFLHWL for 20 min at 37°C, then stained with 7-AAD and either FITC-conjugated anti-CD8 or PE-conjugated anti-CD8β for 30 min on ice, washed twice, and resuspended in PBS. *C*, 2.5×10^5 PBMCs were stained with PerCP-conjugated anti-CD8, 7-AAD, and either FITC-conjugated anti–αβ-TCR, allophycocyanin-conjugated anti-CD56, or PE-conjugated anti–γδ-TCR for 30 min on ice, washed twice, and resuspended in PBS. In *A*, *B*, and *C*, data were acquired using a FACSCalibur flow cytometer and analyzed with FlowJo software.

of CD8αα expression stain with this reagent. Examination of PBMCs from healthy donors revealed that CD8$^+$αβ-TCR$^+$ cells express high levels of CD8, whereas NK and γδ-TCR$^+$ cells express substantially lower levels (Fig. 3*C*). Therefore, increasing the strength of the pMHCI/CD8 interaction allows pMHCI ligand binding at the cell surface that can be mediated through the engagement of either CD8αα or CD8αβ. However, our results suggest that binding is only observed when cells express CD8 at levels above a certain threshold. Importantly, these data demonstrate that TCR expression is not required for cell surface binding of A2/K^b tetramers.

A2/K^b tetramers activate CTLs irrespective of TCR specificity

It is well established that pMHCI tetramers can activate CTLs bearing cognate TCR [reviewed in (35)]. However, previous studies have shown that pMHCI tetrameric binding at the cell surface does not necessarily equate with activation (11, 37). Thus, we next examined whether nonspecific A2/K^b tetramer binding at the cell surface (Figs. 1–3) could activate human CTLs. Initially, we studied the A2-restricted SLYNTVATL-specific CTL clone 003 (23). Consistent with our findings previously stated, both A2 SLYNTVATL and A2/K^b SLYNTVATL tetramers stained 003 CTLs efficiently, as did the noncognate A2/K^b LLFGYPVYV tetramer; no staining was observed with the A2 LLFGYPVYV tetramer (Fig. 4*A*). On ligation, it is known that TCRs are downregulated from the cell surface (38). The cognate A2 tetramer was able to induce significant TCR downregulation, even at tetramer concentrations well below the limits of detection by flow cytometry; no TCR downregulation was observed with the noncognate A2 LLFGYPVYV tetramer (Fig. 4*B*). In contrast, however, both the A2/K^b SLYNTVATL and A2/K^b LLFGYPVYV tetramers induced TCR downregulation, although this occurred to a lesser extent with the noncognate form compared with either of the cognate tetramers (Fig. 4*B*). This TCR downregulation correlated with various functional readouts typical of CTL effector activity, including the production of RANTES (Fig. 4*C*), IFN-γ, and MIP-1β (data not shown). Similar results were observed with SLYNTVATL-specific CTLs bearing an alternative cognate TCR (Fig. 4*D*, 4*E*). Consistent with the staining patterns (Fig. 4*A*), the activation of CTLs by noncognate A2/K^b tetramers was less efficient than that induced by tetramers bearing the agonist peptide (Fig. 4*C–E*).

To dissect this effect further at the single-cell level within a clonal CTL population, we used a flow cytometric assay for degranulation based on the detection of CD107a mobilized on to the cell surface (32). The noncognate A2/K^b tetramer, in this case folded around the GLCTLVAML peptide, induced degranulation in 15% of 003 CTLs at a concentration of 5 μg/ml (Fig. 4*F*); the cognate A2 SLYNTVATL and A2/K^b SLYNTVATL tetramers induced almost 40% degranulation (data not shown). Notably, the cells that degranulated in response to the A2/K^b GLCTLVAML tetramer were contained

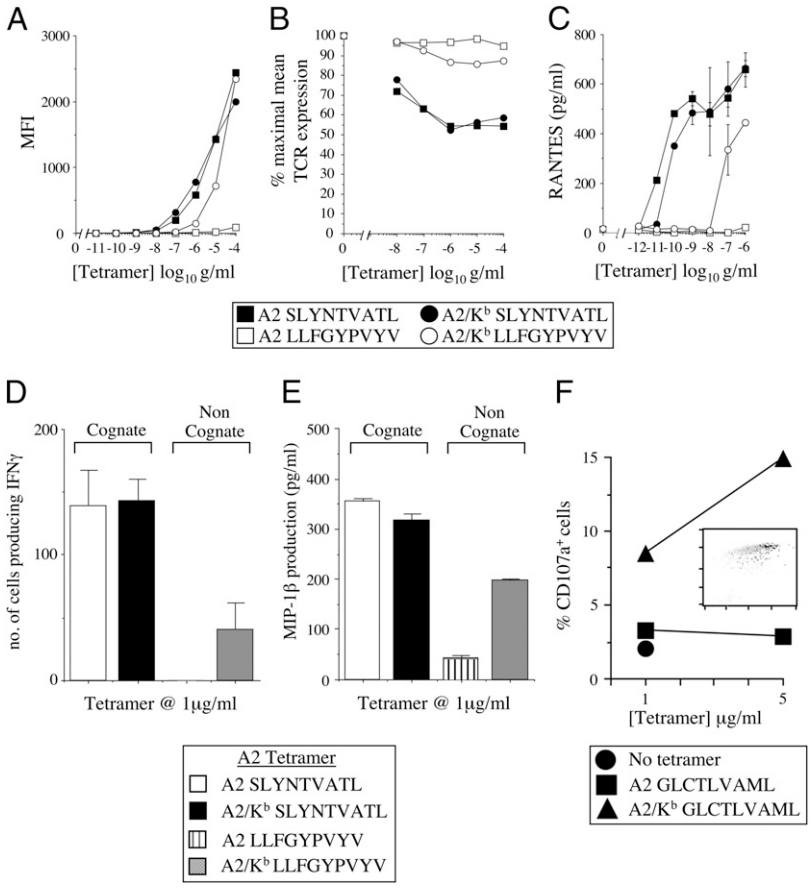

FIGURE 4. A2/K^b tetramers can activate CTLs in the absence of a specific TCR/pMHCI interaction. *A*, 10^5 003 CTLs were suspended in 20 μl PBS and stained with the PE-conjugated tetramers A2 SLYNTVATL, A2/K^b SLYNTVATL, A2 LLFGYPVYV, or A2/K^b LLFGYPVYV at the indicated concentrations and 7-AAD for 20 min at 37°C. Cells were then washed twice and resuspended in PBS. Data were acquired using a FACSCalibur flow cytometer and analyzed with CellQuest software. *B*, 10^5 003 CTLs were suspended in 40 μl R2 with the PE-conjugated tetramers A2 SLYNTVATL, A2/K^b SLYNTVATL, A2 LLFGYPVYV, or A2/K^b LLFGYPVYV at the indicated concentrations for 30 min at 37°C. Cells were subsequently stained with FITC-conjugated anti–αβ-TCR, 7-AAD, and allophycocyanin-conjugated anti-CD8 for 30 min on ice in azide buffer (0.1% azide/2% FCS/PBS). After two washes, data were acquired using a FACSCalibur flow cytometer and analyzed with CellQuest software. *C*, 5×10^5 003 CTLs were incubated with the PE-conjugated tetramers A2 SLYNTVATL, A2/K^b SLYNTVATL, A2 LLFGYPVYV, or A2/K^b LLFGYPVYV at the indicated concentrations. After 4 h at 37°C, supernatants were harvested and assayed for RANTES, IFN-γ and MIP-1β content by ELISA (only RANTES shown). *D*, 2×10^3 868 CTLs were incubated for 4 h at 37°C with 1 μg/ml of the PE-conjugated tetramers A2 SLYNTVATL, A2/K^b SLYNTVATL, A2 LLFGYPVYV, or A2/K^b LLFGYPVYV in an IFN-γ ELISpot assay. *E*, 1.25×10^5 868 CTLs were incubated with 1 μg/ml of the PE-conjugated tetramers A2 SLYNTVATL, A2/K^b SLYNTVATL, A2 LLFGYPVYV, or A2/K^b LLFGYPVYV for 4 h at 37°C. The supernatant was subsequently assayed for MIP-1β content by ELISA. *C–E* show the mean ± SD of two replicate assays. Results similar to *A–E* were also obtained with tetramers conjugated to fluorochromes other than PE (data not shown). *F*, 003 CTLs were incubated with the PE-conjugated tetramers A2 SLYNTVATL, A2/K^b SLYNTVATL, A2 GLCTLVAML, or A2/K^b GLCTLVAML at the indicated concentrations for 4 h at 37°C, then stained with allophycocyanin-conjugated anti-CD8 for 20 min on ice and assayed for CD107a mobilization as described in *Materials and Methods*. The inset plot shows staining for allophycocyanin-conjugated anti-CD8 on the *x*-axis and PE-conjugated A2/K^b GLCTLVAML tetramer (5 μg/ml) on the *y*-axis. Backgated tetramer$^+$CD107a$^+$ cells are shown in black and tetramer$^+$CD107a$^-$ cells are shown in gray. Tetramer^{high}CD8^{high} cells are preferentially activated by the A2/K^b tetramer.

almost exclusively within the tetramer^{high}CD8^{high} population (Fig. 4*F*). Thus, at least to some extent, the strong interaction between A2/K^b and CD8 can bypass the requirement for a specific TCR/pMHCI interaction and nonspecifically activate human CTLs.

Cell surface-expressed A2/K^b activates CTLs in the absence of cognate Ag

To extend our investigation to the effects of cell surface pMHCI presentation, C1R cells were transfected with either A2 or A2/K^b; stable transfectants expressing similar cell surface MHCI densities were selected as targets for further experiments. Target cells expressing either A2 or A2/K^b were incubated overnight with three A2-restricted CTL clones with different peptide specificities (Mel13, 003, and ILA1). Targets that expressed A2 failed to activate any of the CTL clones significantly above background (Fig. 5*A*). Remarkably, however, the A2/K^b targets stimulated Mel13, 003, and ILA1 CTLs to produce significant amounts of MIP-1β in the absence of specific peptide (Fig. 5*A*). A2/K^b targets also elicited substantial levels of TNFα and IFN-γ at titratable E:T ratios (Fig. 5*B*), induced degranulation (Fig. 5*C*), and induced significant levels of killing (data not shown) in the absence of specific TCR/pMHCI interactions.

Cell surface-expressed A2/K^b primes noncognate CTL expansions

Thymic output in healthy A2$^+$ individuals is known to generate a high frequency of naïve CD8$^+$ T cells that can recognize the self-Ag Melan-A$_{26–35}$ (39); this system can be used to examine the priming of CTLs directly ex vivo (40). We exploited these observations to investigate the effect of superenhanced pMHCI/CD8 binding on CTL priming. In priming experiments conducted with C1R target cells, the percentages of CTLs specific for Melan-A$_{26–35}$ that were present after 10 d in culture were related to the context of the pMHCI/CD8 interaction in which the cognate ELAGIGILTV peptide was presented.

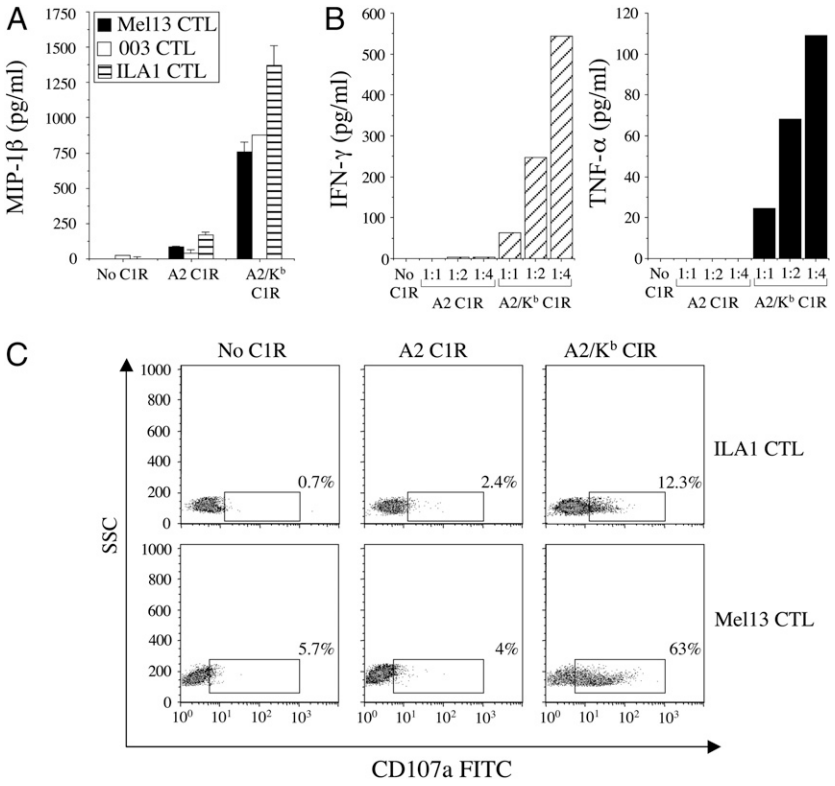

FIGURE 5. Cell surface-expressed A2/K^b activates CTLs in the absence of cognate Ag. *A*, 2.5 × 10^4 Mel13, 003, or ILA1 CTLs were incubated for 12 h at 37°C with 10^5 C1R cells stably transfected to express equal levels of either A2 or A2/K^b at the cell surface. Supernatant was subsequently assayed for MIP-1β content by ELISA. The mean ± SD of two replicate assays is shown. *B*, 2.5 × 10^4 Mel13 CTLs were incubated for 12 h at 37°C with 10^5 C1R cells stably transfected to express either A2 or A2/K^b at the cell surface. Supernatant was assayed for IFN-γ and TNFα content by cytokine bead array. *C*, CD107a expression by ILA1 and Mel13 CTLs after a 12-h incubation at 37°C with C1R cells stably transfected to express either A2 or A2/K^b on the cell surface. For *A–C*, C1R cells were not previously pulsed with peptide.

Thus, in the absence of a pMHCI/CD8 interaction (A2 D227K/T228A C1R targets), only 1.5% of the CD8$^+$ cell population was specific for Melan-A$_{26–35}$; in contrast, 5.6% and 5.7% of the CD8$^+$ population bound the A2 ELAGIGILTV tetramer in the same experiment when priming was conducted with A2 and A2/K^b C1R targets, respectively (Fig. 6). Exposure to A2/K^b C1R targets also resulted in substantial expansions of the total CD8$^+$ population (Fig. 6). Similar results were obtained with multiple donors (data not shown). Thus, target cells that express MHCI molecules with superenhanced CD8-binding properties can induce nonspecific expansions of CD8$^+$ cells in the absence of cognate Ag.

Nonspecific A2/K^b-mediated CTL activation and tetramer staining are not dependent on TCR expression

In earlier experiments, we observed that A2/K^b tetramers bound to the majority of αβ-TCR$^+$CD8$^+$ cells in PBMCs derived from A2$^+$ donors (Fig. 2). To exclude the possibility that this phenomenon was dependent on the presence of A2-restricted TCRs, we conducted staining experiments with A2$^-$ PBMCs. As previously, the A2/K^b ILAKFLHWL tetramer bound nonspecifically to the majority of CD8$^+$ cells (Fig. 7*A*). Furthermore, A2/K^b tetramer binding favored CD8^{high} cells and was abrogated by pretreatment with the anti-CD8 mAb DK25 (Fig. 7*A*). Thus, consistent with the data shown in Fig. 3, nonspecific A2/K^b tetramer binding is a CD8-mediated effect that is not dependent on the presence of A2-restricted TCRs. In addition, we demonstrated in earlier experiments that A2/K^b, both in soluble and cell-associated form, nonspecifically activated A2-restricted CTL (Figs. 4, 5). To confirm that these functional correlates of nonspecific binding were similarly independent of A2-restricted TCR expression, we extended our studies to CTL clones restricted by non-A2 MHCI molecules. In all cases, cell surface-expressed A2/K^b activated CTL clones regardless of restriction element (Fig. 7*B*).

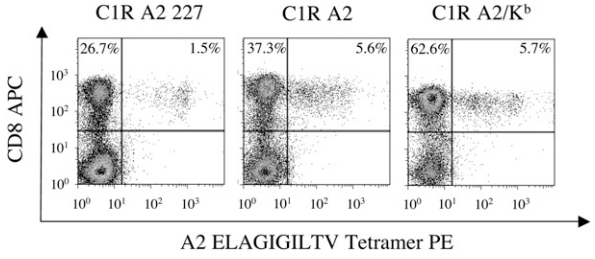

FIGURE 6. Cell surface-expressed A2/K^b primes nonspecific expansion of CD8$^+$ cells. 10^6 A2$^+$ PBMCs were incubated with 2 × 10^5 irradiated A2 D227K/T228A, A2, or A2/K^b C1R cells that had previously been pulsed with 1 μM ELAGIGILTV (Melan-A$_{26–35}$) peptide in R10. From day 3, IL-2 was added in increments to reach a maximum concentration of 200 IU/ml by day 10. Lines were subsequently stained with PE-conjugated A2 ELAGIGILTV tetramer, followed by allophycocyanin-conjugated anti-CD8 and 7-AAD. Data were acquired using a FACSCalibur flow cytometer and analyzed with FlowJo software.

Discussion

CD8 has the potential to engage all pMHCI complexes, both self and foreign, because it binds to largely nonpolymorphic regions of the MHCI molecule. Indeed, recent publications suggest that the ability of CD8 to interact with nonstimulatory pMHCI complexes lowers T cell activation thresholds and enables CTLs to respond to low copy numbers of specific pMHCI (41, 42). It therefore remains unclear how the specificity of TCR recognition is maintained, despite the potential for multiple pMHCI/CD8 interactions at the cell surface. One possibility resides in the fact that the binding of CD8 to MHCI is characterized by very low affinities and extremely rapid kinetics. In this study, we have generated chimeric A2/K^b MHCI molecules that increase the strength of the pMHCI/CD8 interaction by ~15-fold to probe the biophysical and functional significance of the low solution binding affinities observed for the pMHCI/CD8 interaction.

Initially, we examined the effect of superenhanced CD8 binding on pMHCI tetramer binding at the cell surface. Increasing the strength of the pMHCI/CD8 interaction by ~15-fold resulted in the total loss of pMHCI

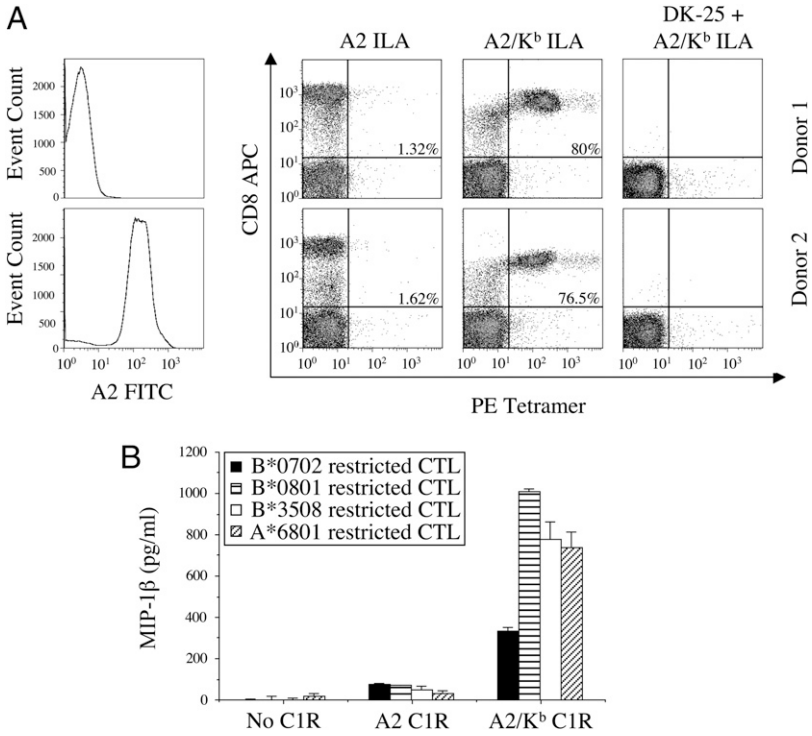

FIGURE 7. Noncognate A2/K^b-mediated CTL activation and tetramer binding is not influenced by MHCI restriction. *A*, 2.5×10^5 PBMCs were suspended in 250 μl FACS buffer (2% FCS/PBS) and stained with FITC-conjugated anti-A2 and 7-AAD for 30 min on ice, then washed twice, and resuspended in PBS. For pMHCI tetramer staining experiments, 2.5×10^5 PBMCs were suspended in 50 μl FACS buffer (2% FCS/PBS) and incubated ± 10 μg/ml unconjugated anti-CD8 for 20 min on ice, then stained with 10 μg/ml of the PE-conjugated tetramers A2 ILAKFLHWL or A2/K^b ILAKFLHWL for 45 min on ice. After washing, cells were subsequently stained with allophycocyanin-conjugated anti-CD8 and 7-AAD, washed again, and resuspended in PBS. Data were acquired using a FACSCalibur flow cytometer and analyzed with FlowJo software. *B*, 2.5×10^4 CTLs were incubated for 12 h at 37°C with 10^5 unpulsed C1R cells expressing either A2 or A2/K^b on the cell surface. The following CTL clones were used: 1) the HLA A*6801-restricted CTL clone c23, specific for the HIV-1 Tat-derived epitope ITKGLGISYGR (residues 38–48); 2) the HLA B*0702-restricted CTL clone KD4, specific for the EBV EBNA3A-derived epitope RPPIFIRRL (residues 379–387); 3) the HLA B*0801-restricted CTL clone LC13, specific for the EBV EBNA3A-derived epitope FLRGRAYGL (residues 339–347); and 4) the HLA B*3508-restricted CTL clone SB27, specific for the EBV BZLF1-derived epitope LPEPLPQGQLTAY (residues 52–64). Supernatant was subsequently assayed for MIP-1β content by ELISA. The mean ± SD of two replicate assays is shown.

tetramer binding specificity. Thus, irrespective of restriction element and the presented peptide, A2/K^b tetramers bound to the surface of all CTL clones examined in this study and to the majority of CTLs present within PBMCs (Figs. 1, 2, 7*A*). In addition, A2/K^b tetramers bound to the cell surface in the absence of TCR expression (Fig. 3) and nonspecific binding was abrogated by pretreatment with an anti-CD8 Ab (Fig. 7*A*), thereby demonstrating that the observed loss of pMHCI tetramer binding specificity was CD8 mediated and TCR independent. These findings indicate that the low solution binding affinities observed for the pMHCI/CD8 interaction are essential for the preservation of pMHCI ligand binding specificity at the cell surface.

It has previously been documented that pMHCI tetramers are efficient activators of cognate CTLs [reviewed in (35)]. However, pMHCI tetramer staining does not necessarily equate with cellular activation. Therefore, we proceeded to examine the ability of A2/K^b tetramers to activate CTL clones. Notably, we found that A2/K^b tetramers activated CTL clones in a nonspecific manner (Fig. 4). Activation resulted in a full range of effector functions, including cytokine/chemokine release, degranulation, and killing. Flow cytometric assessment of degranulation by analysis of CD107a mobilization revealed that CTLs with higher surface expression of CD8 were the cells most likely to activate in response to A2/K^b molecules. This finding led us to examine the effects of cell-surface presented Ag. Strikingly, exposure of PBMCs to C1R target cells bearing A2/K^b molecules caused a general nonspecific expansion of CD8$^+$ cells during the course of the experiment (Fig. 6). Furthermore, A2/K^b C1R cells, unlike their wild-type A2 counterparts, were capable of stimulating effector function in all CTL clones tested regardless of specificity and MHCI restriction (Figs. 5, 7*B*). Although we cannot exclude the possibility that inclusion of the murine α3 domain induces conformational changes at the T cell surface on binding to CD8 that favor noncognate activation, this seems unlikely given that: 1) the TCR binding site remains unaltered (9, 22); 2) a degree of noncognate activation can be observed in long-term assays with nonchimeric human MHCI molecules that exhibit incrementally enhanced CD8 binding (data not shown); and 3) murine and human pMHCI/CD8αα cocrystals exhibit similar binding orientations (14, 43). Furthermore, these results are consistent with the observation that thymus leukemia Ag, which interacts strongly (K_D ~12 μM) with cell surface CD8αα expressed by intraepithelial lymphocytes, can modulate T cell responses independently of the TCR (44–46).

How does a superenhanced pMHCI/CD8 interaction result in nonspecific CTL activation? We have previously demonstrated that an incremental increase in the pMHCI/CD8 interaction (A2 Q115E) results in enhanced immunogenicity of cognate Ags and that this effect is mediated by enhanced early intracellular signal transduction (9, 47). In contrast, the stimulatory properties of A2/K^b molecules exhibited no peptide specificity requirements whatsoever; indeed, cell surface-expressed A2/K^b was shown to activate even non-A2–restricted CTL clones (Fig. 7*B*), thereby confirming that cognate TCR/pMHCI interactions are not required. Combined with the ability of A2/K^b to engage multiple CD8 molecules at the cell surface, these results suggest that A2/K^b cross-links CD8 and induces activation in an "Ab-like" manner. Indeed, this is consistent with previous studies demonstrating that Ab-induced CD8

cross-linking can induce T cell signaling (48, 49) and elicit downstream effector functions, such as chemokine release (50); such effects are predictable given that the CD8α tail is coupled to p56lck, an essential component of the early intracellular signaling pathway (10). It is interesting to note that the murine pMHCI/CD8 interaction is significantly stronger (K_D ~30 μM) than the equivalent human interaction (K_D ~150 μM) (11), but does not result in noncognate CTL activation. It is therefore likely that a pMHCI/CD8 interaction affinity threshold exists for the maintenance of CTL activation specificity. The strength of the murine pMHCI/CD8 interaction is 3-fold weaker than the strength of the interaction measured between A2/K^b and human CD8, thereby still operating at a level below this threshold.

In summary, we used chimeric MHCI molecules that exhibit a superenhanced interaction with CD8 to probe the physical and functional significance of the low solution binding affinities previously described for the pMHCI/CD8 interaction. We found that increasing the strength of the pMHCI/CD8 interaction by ~15-fold resulted in: 1) total loss of pMHCI binding specificity at the cell surface; 2) noncognate pMHCI tetramer-mediated activation; and 3) nonspecific activation and proliferation triggered by cell surface-expressed pMHCI molecules. Thus, the low solution binding affinity of the pMHCI/CD8 interaction is essential for the preservation of pMHCI ligand binding specificity at the cell surface and its attendant functional repercussions.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Davis, M. M., J. J. Boniface, Z. Reich, D. Lyons, J. Hampl, B. Arden, and Y. Chien. 1998. Ligand recognition by alpha beta T cell receptors. *Annu. Rev. Immunol.* 16: 523–544.
2. Rudolph, M. G., and I. A. Wilson. 2002. The specificity of TCR/pMHC interaction. *Curr. Opin. Immunol.* 14: 52–65.
3. Zamoyska, R. 1998. CD4 and CD8: modulators of T-cell receptor recognition of antigen and of immune responses? *Curr. Opin. Immunol.* 10: 82–87.
4. Holler, P. D., and D. M. Kranz. 2003. Quantitative analysis of the contribution of TCR/pepMHC affinity and CD8 to T cell activation. *Immunity* 18: 255–264.
5. Gakamsky, D. M., I. F. Luescher, A. Pramanik, R. B. Kopito, F. Lemonnier, H. Vogel, R. Rigler, and I. Pecht. 2005. CD8 kinetically promotes ligand binding to the T-cell antigen receptor. *Biophys. J.* 89: 2121–2133.
6. Laugel, B., H. A. van den Berg, E. Gostick, D. K. Cole, L. Wooldridge, J. Boulter, A. Milicic, D. A. Price, and A. K. Sewell. 2007. Different T cell receptor affinity thresholds and CD8 coreceptor dependence govern cytotoxic T lymphocyte activation and tetramer binding properties. *J. Biol. Chem.* 282: 23799–23810.
7. van den Berg, H. A., L. Wooldridge, B. Laugel, and A. K. Sewell. 2007. Coreceptor CD8-driven modulation of T cell antigen receptor specificity. *J Theor Biol* 249: 395-408.
8. Luescher, I. F., E. Vivier, A. Layer, J. Mahiou, F. Godeau, B. Malissen, and P. Romero. 1995. CD8 modulation of T-cell antigen receptor-ligand interactions on living cytotoxic T lymphocytes. *Nature* 373: 353–356.
9. Wooldridge, L., H. A. van den Berg, M. Glick, E. Gostick, B. Laugel, S. L. Hutchinson, A. Milicic, J. M. Brenchley, D. C. Douek, D. A. Price, and A. K. Sewell. 2005. Interaction between the CD8 coreceptor and major histocompatibility complex class I stabilizes T cell receptor-antigen complexes at the cell surface. *J. Biol. Chem.* 280: 27491–27501.
10. Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. *Cell* 55: 301–308.
11. Purbhoo, M. A., J. M. Boulter, D. A. Price, A. L. Vuidepot, C. S. Hourigan, P. R. Dunbar, K. Olson, S. J. Dawson, R. E. Phillips, B. K. Jakobsen, et al. 2001. The human CD8 coreceptor effects cytotoxic T cell activation and antigen sensitivity primarily by mediating complete phosphorylation of the T cell receptor zeta chain. *J. Biol. Chem.* 276: 32786–32792.
12. Arcaro, A., C. Grégoire, T. R. Bakker, L. Baldi, M. Jordan, L. Goffin, N. Boucheron, F. Wurm, P. A. van der Merwe, B. Malissen, and I. F. Luescher. 2001. CD8beta endows CD8 with efficient coreceptor function by coupling T cell receptor/CD3 to raft-associated CD8/p56(lck) complexes. *J. Exp. Med.* 194: 1485–1495.
13. Arcaro, A., C. Grégoire, N. Boucheron, S. Stotz, E. Palmer, B. Malissen, and I. F. Luescher. 2000. Essential role of CD8 palmitoylation in CD8 coreceptor function. *J. Immunol.* 165: 2068–2076.
14. Gao, G. F., J. Tormo, U. C. Gerth, J. R. Wyer, A. J. McMichael, D. I. Stuart, J. I. Bell, E. Y. Jones, and B. K. Jakobsen. 1997. Crystal structure of the complex between human CD8alpha(alpha) and HLA-A2. *Nature* 387: 630–634.
15. Wyer, J. R., B. E. Willcox, G. F. Gao, U. C. Gerth, S. J. Davis, J. I. Bell, P. A. van der Merwe, and B. K. Jakobsen. 1999. T cell receptor and coreceptor CD8 alphaalpha bind peptide-MHC independently and with distinct kinetics. *Immunity* 10: 219–225.
16. Gao, G. F., B. E. Willcox, J. R. Wyer, J. M. Boulter, C. A. O'Callaghan, K. Maenaka, D. I. Stuart, E. Y. Jones, P. A. Van Der Merwe, J. I. Bell, and B. K. Jakobsen. 2000. Classical and nonclassical class I major histocompatibility complex molecules exhibit subtle conformational differences that affect binding to CD8alphaalpha. *J. Biol. Chem.* 275: 15232–15238.
17. Dustin, M. L., D. E. Golan, D. M. Zhu, J. M. Miller, W. Meier, E. A. Davies, and P. A. van der Merwe. 1997. Low affinity interaction of human or rat T cell adhesion molecule CD2 with its ligand aligns adhering membranes to achieve high physiological affinity. *J. Biol. Chem.* 272: 30889–30898.
18. Cole, D. K., N. J. Pumphrey, J. M. Boulter, M. Sami, J. I. Bell, E. Gostick, D. A. Price, G. F. Gao, A. K. Sewell, and B. K. Jakobsen. 2007. Human TCR-binding affinity is governed by MHC class restriction. *J. Immunol.* 178: 5727–5734.
19. Varela-Rohena, A., P. E. Molloy, S. M. Dunn, Y. Li, M. M. Suhoski, R. G. Carroll, A. Milicic, T. Mahon, D. H. Sutton, B. Laugel, et al. 2008. Control of HIV-1 immune escape by CD8 T cells expressing enhanced T-cell receptor. *Nat. Med.* 14: 1390–1395.
20. Stone, J. D., A. S. Chervin, and D. M. Kranz. 2009. T-cell receptor binding affinities and kinetics: impact on T-cell activity and specificity. *Immunology* 126: 165–176.
21. Hutchinson, S. L., L. Wooldridge, S. Tafuro, B. Laugel, M. Glick, J. M. Boulter, B. K. Jakobsen, D. A. Price, and A. K. Sewell. 2003. The CD8 T cell coreceptor exhibits disproportionate biological activity at extremely low binding affinities. *J. Biol. Chem.* 278: 24285–24293.
22. Choi, E. M., J. L. Chen, L. Wooldridge, M. Salio, A. Lissina, N. Lissin, I. F. Hermans, J. D. Silk, F. Mirza, M. J. Palmowski, et al. 2003. High avidity antigen-specific CTL identified by CD8-independent tetramer staining. *J. Immunol.* 171: 5116–5123.
23. Sewell, A. K., G. C. Harcourt, P. J. Goulder, D. A. Price, and R. E. Phillips. 1997. Antagonism of cytotoxic T lymphocyte-mediated lysis by natural HIV-1 altered peptide ligands requires simultaneous presentation of agonist and antagonist peptides. *Eur. J. Immunol.* 27: 2323–2329.
24. Purbhoo, M. A., Y. Li, D. H. Sutton, J. E. Brewer, E. Gostick, G. Bossi, B. Laugel, R. Moysey, E. Baston, N. Liddy, et al. 2007. The HLA A*0201-restricted hTERT(540-548) peptide is not detected on tumor cells by a CTL clone or a high-affinity T-cell receptor. *Mol. Cancer Ther.* 6: 2081–2091.
25. Gostick, E., D. K. Cole, S. L. Hutchinson, L. Wooldridge, S. Tafuro, B. Laugel, A. Lissina, A. Oxenius, J. M. Boulter, D. A. Price, and A. K. Sewell. 2007. Functional and biophysical characterization of an HLA-A*6801-restricted HIV-specific T cell receptor. *Eur. J. Immunol.* 37: 479–486.
26. Burrows, S. R., S. L. Silins, D. J. Moss, R. Khanna, I. S. Misko, and V. P. Argaet. 1995. T cell receptor repertoire for a viral epitope in humans is diversified by tolerance to a background major histocompatibility complex antigen. *J. Exp. Med.* 182: 1703–1715.
27. Kjer-Nielsen, L., C. S. Clements, A. W. Purcell, A. G. Brooks, J. C. Whisstock, S. R. Burrows, J. McCluskey, and J. Rossjohn. 2003. A structural basis for the selection of dominant alphabeta T cell receptors in antiviral immunity. *Immunity* 18: 53–64.
28. Tynan, F. E., S. R. Burrows, A. M. Buckle, C. S. Clements, N. A. Borg, J. J. Miles, T. Beddoe, J. C. Whisstock, M. C. Wilce, S. L. Silins, et al. 2005. T cell receptor recognition of a 'super-bulged' major histocompatibility complex class I-bound peptide. *Nat. Immunol.* 6: 1114–1122.
29. Tynan, F. E., N. A. Borg, J. J. Miles, T. Beddoe, D. El-Hassen, S. L. Silins, W. J. van Zuylen, A. W. Purcell, L. Kjer-Nielsen, J. McCluskey, et al. 2005. High resolution structures of highly bulged viral epitopes bound to major histocompatibility complex class I. Implications for T-cell receptor engagement and T-cell immunodominance. *J. Biol. Chem.* 280: 23900–23909.
30. Willemsen, R., C. Ronteltap, M. Heuveling, R. Debets, and R. Bolhuis. 2005. Redirecting human CD4+ T lymphocytes to the MHC class I-restricted melanoma antigen MAGE-A1 by TCR alphabeta gene transfer requires CD8alpha. *Gene Ther.* 12: 140–146.
31. Willemsen, R. A., Z. Sebestyén, C. Ronteltap, C. Berrevoets, J. Drexhage, and R. Debets. 2006. CD8 alpha coreceptor to improve TCR gene transfer to treat melanoma: down-regulation of tumor-specific production of IL-4, IL-5, and IL-10. *J. Immunol.* 177: 991–998.
32. Betts, M. R., J. M. Brenchley, D. A. Price, S. C. De Rosa, D. C. Douek, M. Roederer, and R. A. Koup. 2003. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J. Immunol. Methods* 281: 65–78.
33. Choi, E. M., M. Palmowski, J. Chen, and V. Cerundolo. 2002. The use of chimeric A2K(b) tetramers to monitor HLA A2 immune responses in HLA A2 transgenic mice. *J. Immunol. Methods* 268: 35–41.
34. Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274: 94–96.
35. Wooldridge, L., A. Lissina, D. K. Cole, H. A. van den Berg, D. A. Price, and A. K. Sewell. 2009. Tricks with tetramers: how to get the most from multimeric peptide-MHC. *Immunology* 126: 147–164.

36. Burrows, S. R., N. Kienzle, A. Winterhalter, M. Bharadwaj, J. D. Altman, and A. Brooks. 2000. Peptide-MHC class I tetrameric complexes display exquisite ligand specificity. *J. Immunol.* 165: 6229–6234.
37. Schott, E., and H. L. Ploegh. 2002. Mouse MHC class I tetramers that are unable to bind to CD8 reveal the need for CD8 engagement in order to activate naive CD8 T cells. *Eur. J. Immunol.* 32: 3425–3434.
38. Valitutti, S., S. Müller, M. Cella, E. Padovan, and A. Lanzavecchia. 1995. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* 375: 148–151.
39. Zippelius, A., M. J. Pittet, P. Batard, N. Rufer, M. de Smedt, P. Guillaume, K. Ellefsen, D. Valmori, D. Liénard, J. Plum, et al. 2002. Thymic selection generates a large T cell pool recognizing a self-peptide in humans. *J. Exp. Med.* 195: 485–494.
40. Salio, M., M. J. Palmowski, A. Atzberger, I. F. Hermans, and V. Cerundolo. 2004. CpG-matured murine plasmacytoid dendritic cells are capable of in vivo priming of functional CD8 T cell responses to endogenous but not exogenous antigens. *J. Exp. Med.* 199: 567–579.
41. Yachi, P. P., J. Ampudia, N. R. Gascoigne, and T. Zal. 2005. Nonstimulatory peptides contribute to antigen-induced CD8-T cell receptor interaction at the immunological synapse. *Nat. Immunol.* 6: 785–792.
42. Anikeeva, N., T. Lebedeva, A. R. Clapp, E. R. Goldman, M. L. Dustin, H. Mattoussi, and Y. Sykulev. 2006. Quantum dot/peptide-MHC biosensors reveal strong CD8-dependent cooperation between self and viral antigens that augment the T cell response. *Proc. Natl. Acad. Sci. USA* 103: 16846–16851.
43. Kern, P. S., M. K. Teng, A. Smolyar, J. H. Liu, J. Liu, R. E. Hussey, R. Spoerl, H. C. Chang, E. L. Reinherz, and J. H. Wang. 1998. Structural basis of CD8 coreceptor function revealed by crystallographic analysis of a murine CD8alphaalpha ectodomain fragment in complex with H-2Kb. *Immunity* 9: 519–530.
44. Leishman, A. J., O. V. Naidenko, A. Attinger, F. Koning, C. J. Lena, Y. Xiong, H. C. Chang, E. Reinherz, M. Kronenberg, and H. Cheroutre. 2001. T cell responses modulated through interaction between CD8alphaalpha and the nonclassical MHC class I molecule, TL. *Science* 294: 1936–1939.
45. Tsujimura, K., Y. Obata, E. Kondo, K. Nishida, Y. Matsudaira, Y. Akatsuka, K. Kuzushima, and T. Takahashi. 2003. Thymus leukemia antigen (TL)-specific cytotoxic T lymphocytes recognize the alpha1/alpha2 domain of TL free from antigenic peptides. *Int. Immunol.* 15: 1319–1326.
46. Cole, D. K., and G. F. Gao. 2004. CD8: adhesion molecule, co-receptor and immunomodulator. *Cell. Mol. Immunol.* 1: 81–88.
47. Wooldridge, L., A. Lissina, J. Vernazza, E. Gostick, B. Laugel, S. L. Hutchinson, F. Mirza, P. R. Dunbar, J. M. Boulter, M. Glick, et al. 2007. Enhanced immunogenicity of CTL antigens through mutation of the CD8 binding MHC class I invariant region. *Eur. J. Immunol.* 37: 1323–1333.
48. Grebe, K. M., R. L. Clarke, and T. A. Potter. 2004. Ligation of CD8 leads to apoptosis of thymocytes that have not undergone positive selection. *Proc. Natl. Acad. Sci. USA* 101: 10410–10415.
49. Clarke, R. L., S. Thiemann, Y. Refaeli, G. Werlen, and T. A. Potter. 2009. A new function for LAT and CD8 during CD8-mediated apoptosis that is independent of TCR signal transduction. *Eur. J. Immunol.* 39: 1619–1631.
50. Wooldridge, L., S. L. Hutchinson, E. M. Choi, A. Lissina, E. Jones, F. Mirza, P. R. Dunbar, D. A. Price, V. Cerundolo, and A. K. Sewell. 2003. Anti-CD8 antibodies can inhibit or enhance peptide-MHC class I (pMHCI) multimer binding: this is paralleled by their effects on CTL activation and occurs in the absence of an interaction between pMHCI and CD8 on the cell surface. *J. Immunol.* 171: 6650–6660.