

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

Linda Wooldridge,^{*,1} Mathew Clement,^{*,1} Anna Lissina,^{*} Emily S. J. Edwards,^{*} Kristin Ladell,^{*} Julia Ekeruche,^{*} Rachel E. Hewitt,[†] Bruno Laugel,^{*} Emma Gostick,^{*} David K. Cole,^{*} Reno Debets,[‡] Cor Berrevoets,[‡] John J. Miles,^{*,§} Scott R. Burrows,[§] David A. Price,^{*} and Andrew K. Sewell^{*}

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: 000–000.

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 $\alpha 1$ and $\alpha 2$ 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 $\alpha\beta$ T cell biology. The pMHCI/CD8 interaction is characterized by very low solution affinities ($K_D \sim 150 \mu\text{M}$) and rapid kinetics ($K_{\text{off}} \sim 18 \text{ s}^{-1}$) (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 ($K_D = 60\text{--}90 \mu\text{M}$) (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 ($K_D \sim 10 \mu\text{M}$) are able to activate CTL in the absence of a specific TCR/pMHCI interaction. Thus, the biophysical

^{*}Department of Infection, Immunity, and Biochemistry, Cardiff University, Cardiff; [†]Micronutrient Status Research Section, Medical Research Council Human Nutrition Research, Cambridge, United Kingdom; [‡]Laboratory of Experimental Tumor Immunology, Department of Medical Oncology, Erasmus Medical Center-Daniel den Hoed Cancer Center, Rotterdam, The Netherlands; and [§]Division of Immunology, Queensland Institute of Medical Research, Brisbane, Australia

¹L.W. and M.C. contributed equally to this manuscript.

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Address correspondence and reprint requests to Dr. Linda Wooldridge, Henry Wellcome Building, Heath Park, Cardiff University, Cardiff, CF144XN, U.K. E-mail address: wooldridgel@cardiff.ac.uk

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 LPEPLPQQQLTAY (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-CD8 α cells were manufactured by introducing pBullet-human CD8 α (30, 31) into 293T cells using vesicular stomatitis virus-pseudotyped Moloney murine leukemia virus particles. The 293T-CD8 α 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 re-fold 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), NLPVMVATV (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 CD8 β (clone 2ST8.5H7; Beckman Coulter, Fullerton, CA), PerCP-conjugated anti-human CD3 (clone SK7; BD Biosciences), FITC-conjugated anti-human $\alpha\beta$ -TCR (clone BMA 031; Serotec, Oxford, U.K.), FITC-conjugated or PE-conjugated anti-human $\gamma\delta$ -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 CD8 α -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- $\alpha\beta$ -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-1 β , 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 \pm 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 (Golgi-Stop; 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

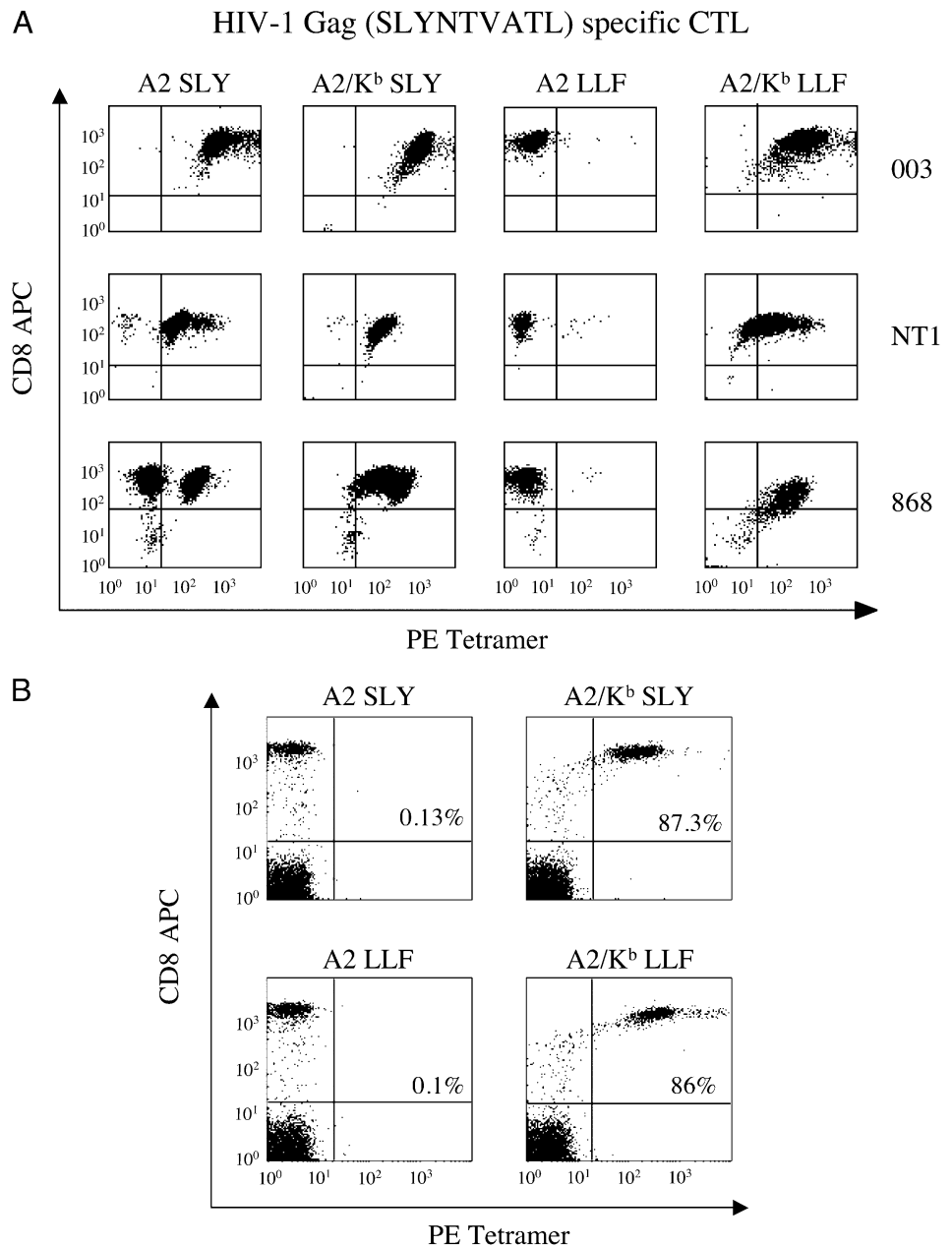
Transfected C1R cells were pulsed with 1 µM ELAGIGILTV (Melan-A_{26–35}) peptide for 90 min, irradiated, and washed once in RPMI 1640 medium. Pulsed, irradiated C1R cells (2×10^5) were incubated with 10^6 fresh A2⁺ human PBMCs in R10; 200 IU/ml IL-2 was added on day 3. CD8⁺ cells specific for Melan-A_{26–35} 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 $\alpha 1/\alpha 2$ peptide binding platform of A2 and the $\alpha 3$ 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 $\alpha 3$ 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 $\beta 2m$ interacts strongly with human CD8 ($K_D \sim 10$ µM, compared with A2 that binds to CD8 with a $K_D \sim 150$ µM) but exhibits unaltered A2-restricted TCR binding properties (9, 22). Thus, fusing the murine $\alpha 3$ domain with A2 $\alpha 1/\alpha 2$ domains increases

FIGURE 1. The exquisite specificity of pMHC/CD8 interaction is lost when the strength of the pMHC/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 Gag₇₇₋₈₅, were stained with 1 μ g of the PE-conjugated tetramers A2 SLYNTVATL, A2/K^b SLYNTVATL, A2 LLFGYPVYV, or A2/K^b LLFGYPVYV in 20 μ l 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 μ l FACS buffer (2% FCS/PBS), then stained with 1 μ g 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 pMHC/CD8 interaction by ~15-fold without affecting the TCR/pMHC interaction.

Superenhanced CD8 binding results in nonspecific pMHC ligand interactions

Monomeric pMHC complexes cannot be used to examine TCR/pMHC 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 pMHC 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 pMHC ligand interactions at the cell surface.

Wild-type pMHC tetrameric reagents bearing cognate peptide stained three distinct A2-restricted CTLs specific for SLYNTVATL

(HIV-1 p17 Gag₇₇₋₈₅), each expressing a different TCR (19, 22, 23) (Fig. 1A). Noncognate A2 LLFGYPVYV (HILV-1 Tax₁₁₋₁₉) 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. 1A). 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. 1B). In contrast, both the A2/K^b SLYNTVATL and A2/K^b LLFGYPVYV tetramers stained >85% of CD8⁺ cells in PBMCs (Fig. 1B); similar data were obtained with A2/K^b GLCTLVAML (EBV BMLF1₂₅₉₋₂₆₇) and A2/K^b NLVPMVATV (CMV pp65₄₉₅₋₅₀₃) tetramers (data not shown). Taken together, these data indicate that the exquisite specificity of tetrameric pMHC reagents is lost when the strength of the pMHC/CD8

interaction is increased by ~ 15 -fold. Thus, the low solution affinities of the wild-type pMHC/CD8 interaction are required to maintain pMHC 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 $\sim 80\%$ of the CD8 α^+ population in peripheral blood (Fig. 1B). Although CD8 α is predominantly found on the surface of $\alpha\beta$ -TCR⁺ CTLs, it is also found on the surface of other lymphocytes, most notably some $\gamma\delta$ T cells and NK cells. We therefore sought to determine the identity of the CD8 α^+ cells that stain with A2/K^b tetramers. Staining of fresh ex vivo PBMCs isolated from healthy A2⁺ donors revealed that CD8 α was expressed on $\sim 39\%$, 54% , and 32% of the $\alpha\beta$ -TCR⁺, NK cells, and $\gamma\delta$ -TCR⁺ populations, respectively, with some variation between donors (Fig. 2A). The majority of $\gamma\delta$ -TCR⁺ ($\sim 93.6\%$) and NK cells ($\sim 77\%$) failed to stain with the A2/K^b ILAKFLHWL (hTERT₅₄₀₋₅₄₈) tetramer and no significant binding was observed with the corresponding A2 tetramer (Fig. 2B). However, the vast majority of

$\alpha\beta$ -TCR⁺/CD8⁺ cells within the lymphocyte population stained nonspecifically with the A2/K^b ILAKFLHWL tetramer (Fig. 2C).

We hypothesized that most $\gamma\delta$ -TCR⁺ cells and NK cells might fail to bind A2/K^b tetramers because they express the CD8 $\alpha\alpha$ homodimer rather than the CD8 $\alpha\beta$ heterodimer, which is expressed on the surface of CTLs. Thus, we generated a 293T cell line that expressed CD8 $\alpha\alpha$ (Fig. 3A) 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 CD8 $\alpha\alpha^+$ 293 T cell transfectants (Fig. 3A, 3B); no binding was observed in the absence of CD8 $\alpha\alpha$ surface expression (Fig. 3A). Thus, A2/K^b tetramers are capable of binding to cell surface CD8 $\alpha\alpha$.

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

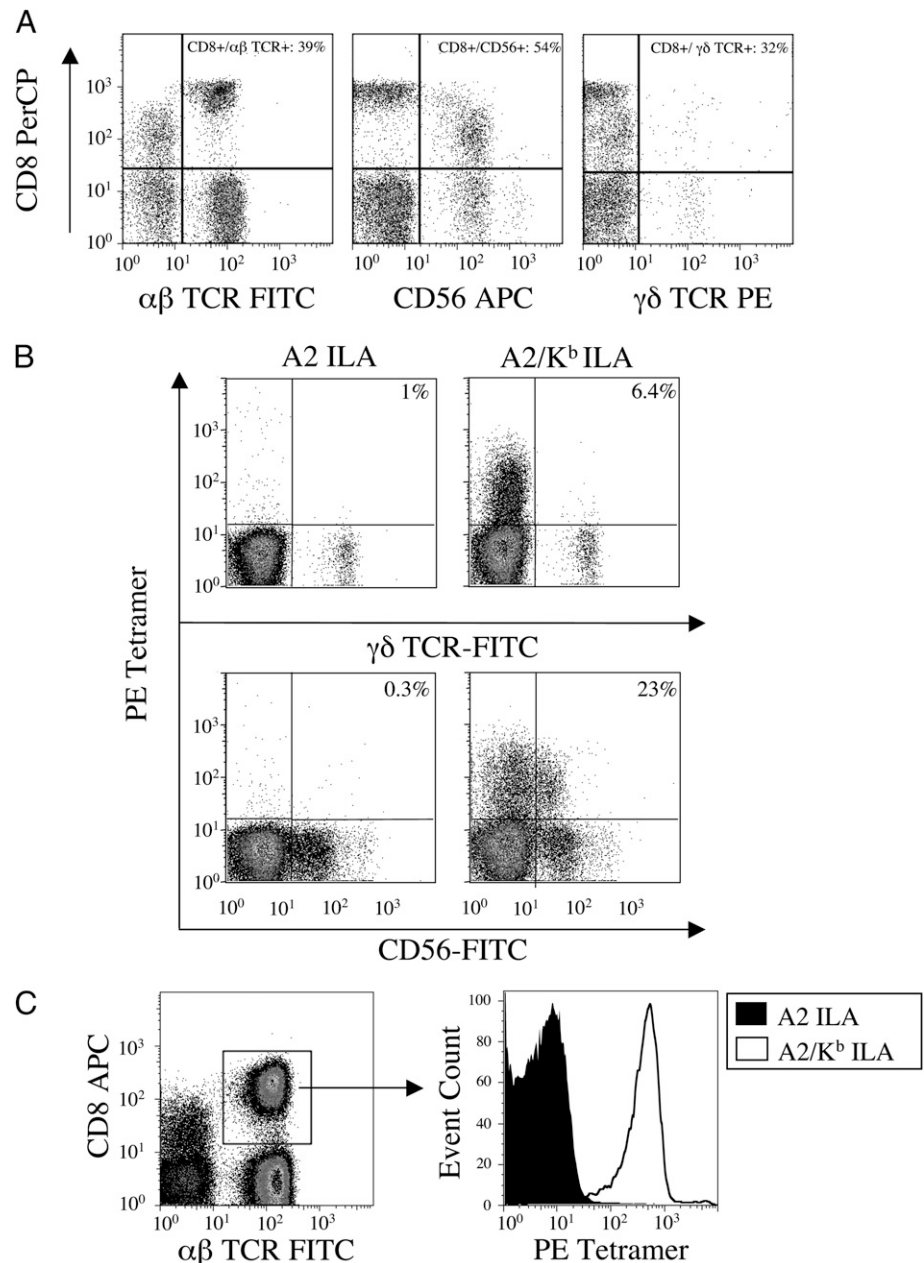


FIGURE 2. A2/K^b tetramers bind the majority of CTLs in peripheral blood. *A*, 2.5×10^5 PBMCs from an A2⁺ donor were stained with PerCP-conjugated anti-CD8, 7-AAD, and either FITC-conjugated anti- $\alpha\beta$ -TCR, allophycocyanin-conjugated anti-CD56 or PE-conjugated anti- $\gamma\delta$ -TCR for 30 min on ice, washed twice, and resuspended in PBS. *B*, 2.5×10^5 A2⁺ PBMCs were stained with 10 $\mu\text{g/ml}$ 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- $\gamma\delta$ -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 $\mu\text{g/ml}$ 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- $\alpha\beta$ -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.

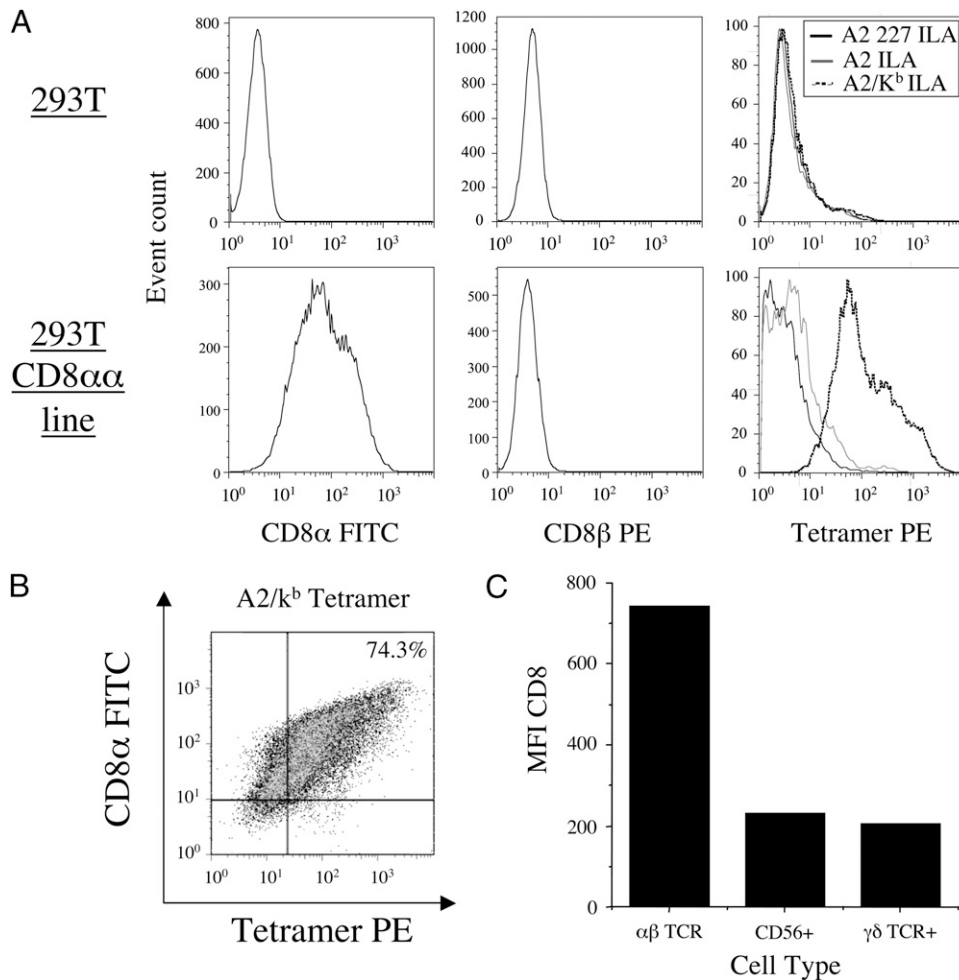


FIGURE 3. Nonspecific A2/K^b tetramer binding is influenced by CD8 cell surface density. *A* and *B*, 2×10^5 293T cells were incubated $\pm 10 \mu\text{g/ml}$ of the PE-conjugated tetramers A2 D227K/T228A ILAKFLHWL, A2 ILAKFLHWL, or A2/K^b ILAKFLHWL for 20 min at 37°C, then stained with 7-AAD and either 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. 3C). Therefore, increasing the strength of the pMHC/CD8 interaction allows pMHC 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 pMHC tetramers can activate CTLs bearing cognate TCR [reviewed in (35)]. However, previous studies have shown that pMHC 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. 4A). 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. 4B). 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. 4B). This TCR downregulation correlated with various functional readouts typical of CTL effector activity, including the production of RANTES (Fig. 4C), IFN-γ, and MIP-1β (data not shown). Similar results were observed with SLYNTVATL-specific CTLs bearing an alternative cognate TCR (Fig. 4D, 4E). Consistent with the staining patterns (Fig. 4A), the activation of CTLs by noncognate A2/K^b tetramers was less efficient than that induced by tetramers bearing the agonist peptide (Fig. 4C–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. 4F); 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

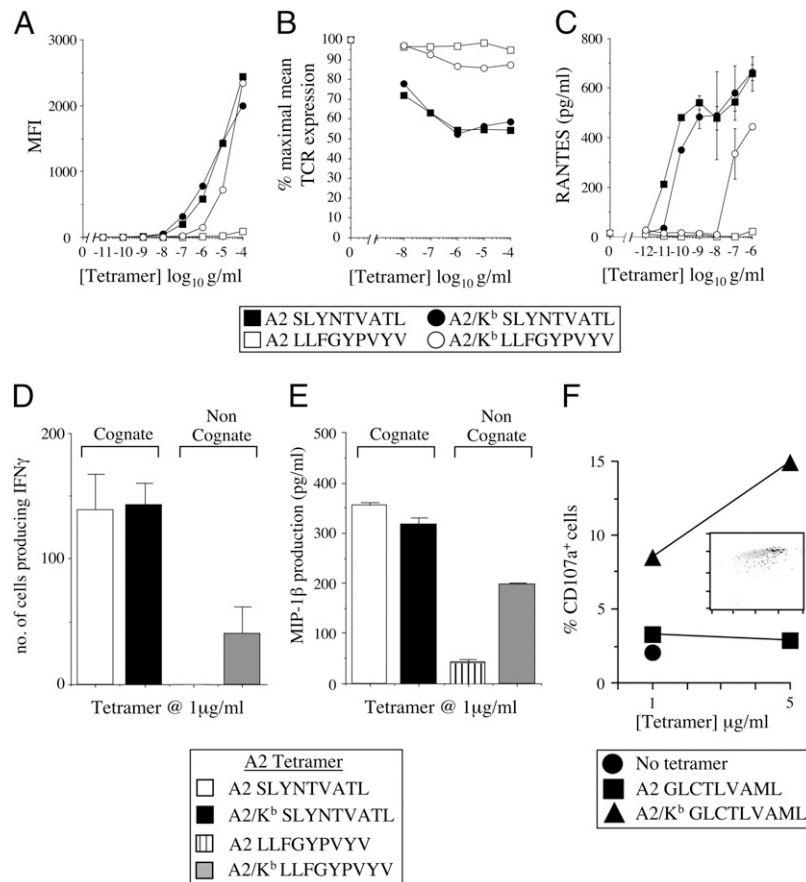


FIGURE 4. A2/K^b tetramers can activate CTLs in the absence of a specific TCR/pMHCI interaction. *A*, 10^5 003 CTLs were suspended in 20 μ l PBS and stained with the PE-conjugated tetramers A2 SLYNTVATL, A2/K^b SLYNTVATL, A2 LLFGYPVYV, or A2/K^b LLFGYPVYV at the indicated concentrations and 7-AAD for 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 μ l 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- α -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-1 β content by ELISA (only RANTES shown). *D*, 2×10^3 868 CTLs were incubated for 4 h at 37°C with 1 μ g/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 μ g/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-1 β content by ELISA. Panels (*C*–*E*) show the mean \pm 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 μ g/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

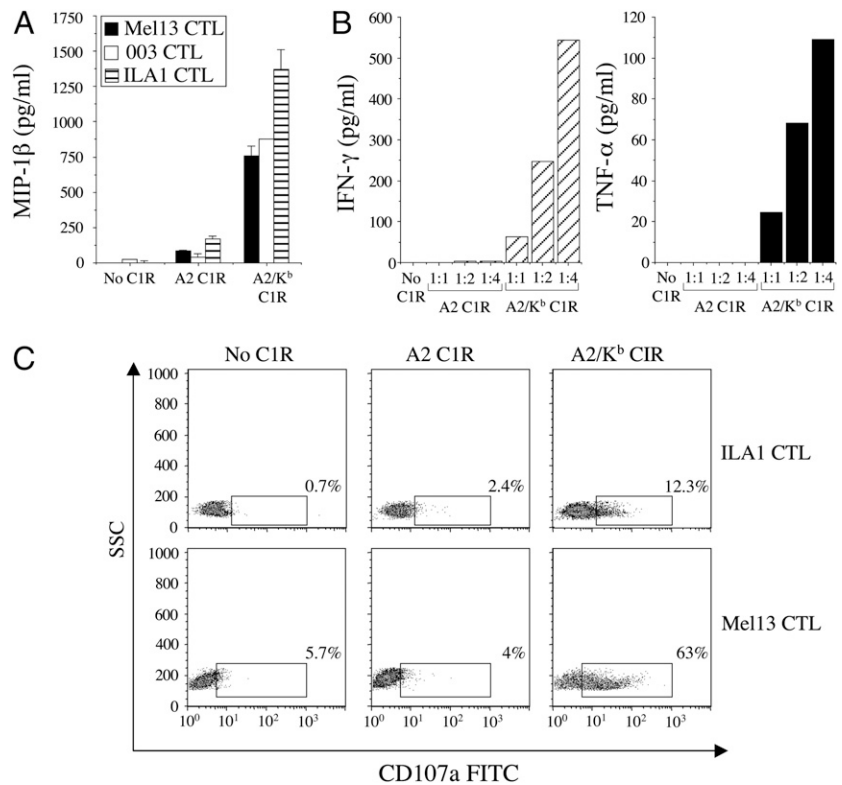
To extend our investigation to the effects of cell surface pMHCI presentation, C1R cells were transfected with either A2 or A2/K^b; stable transfectants expressing similar cell surface MHCII densities were selected as targets for further experiments. Target cells expressing either A2 or A2/K^b were incubated 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-1 β in the absence of specific peptide (Fig. 5*A*). A2/K^b targets also elicited substantial levels of TNF α 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 pMHCII/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 pMHCII/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-1 β content by ELISA. The mean \pm 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 TNF α 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 pMHC/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 MHC I 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 $\alpha\beta$ -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. 7A). Furthermore, A2/K^b tetramer binding favored CD8^{high} cells and was abrogated by pretreatment with the anti-CD8 mAb DK25 (Fig. 7A). 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 MHC I molecules. In all cases, cell surface-expressed A2/K^b activated CTL clones regardless of restriction element (Fig. 7B).

Discussion

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

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

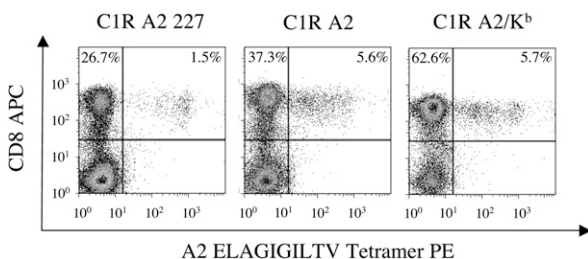


FIGURE 6. Cell surface-expressed A2/K^b primes nonspecific expansion of CD8⁺ cells. 10^6 A2⁺ PBMCs were incubated with 2×10^5 irradiated A2 D227K/T228A, A2, or A2/K^b C1R cells that had previously been pulsed with 1 μ M ELAGIGILTV (Melan-A_{26–35}) peptide in R10. From day 3, IL-2 was added in increments 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.

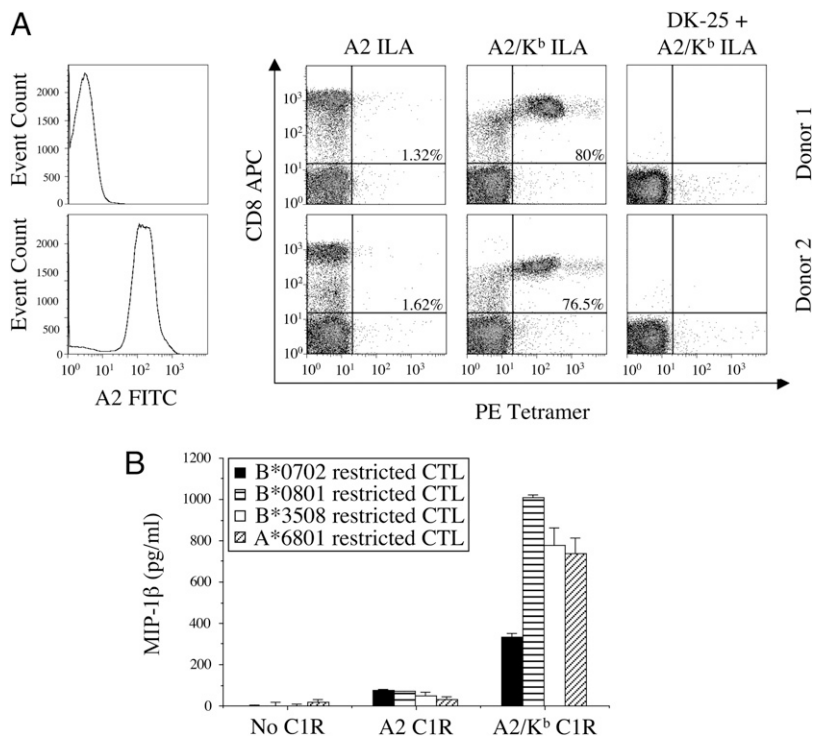


FIGURE 7. Noncognate A2/K^b-mediated CTL activation and tetramer binding is not influenced by MHCI restriction. *A*, 2.5×10^5 PBMCs were suspended in 250 μ l FACS buffer (2% FCS/PBS) and stained with FITC-conjugated anti-A2 and 7-AAD for 30 min on ice, then washed twice, and resuspended in PBS. For pMHCI tetramer staining experiments, 2.5×10^5 PBMCs were suspended in 50 μ l FACS buffer (2% FCS/PBS) and incubated $\pm 10 \mu$ g/ml unconjugated anti-CD8 for 20 min on ice, then stained with 10 μ g/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 CIR 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 RPPFIRRL (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 LPEPLPQQQLTAY (residues 52–64). Supernatant was subsequently assayed for MIP-1 β content by ELISA. The mean \pm 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, 7A). In addition, A2/K^b tetramers bound to the cell surface in the absence of TCR expression (Fig. 3) and non-specific binding was abrogated by pretreatment with an anti-CD8 Ab (Fig. 7A), 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 CIR target cells bearing A2/K^b molecules caused a general non-specific expansion of CD8⁺ cells during the course of the experiment (Fig. 6). Furthermore, A2/K^b CIR 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, 7B). Although we cannot exclude the possibility that inclusion of the murine $\alpha 3$ 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/CD8 $\alpha\alpha$ cocrystals exhibit similar binding orientations (14, 43). Furthermore, these results are consistent with the observation that thymus leukemia Ag, which interacts strongly ($K_D \sim 12 \mu$ M) with cell surface CD8 $\alpha\alpha$ 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. 7B), 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 CD8 α 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 ($K_D \sim 30 \mu\text{M}$) than the equivalent human interaction ($K_D \sim 150 \mu\text{M}$) (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.

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