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


*J Immunol* published online 26 February 2010
http://www.jimmunol.org/content/early/2010/02/26/jimmunol.0902398
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,*８ 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 (MHC1) 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 MHC1 [pMHC1]). The TCR/pMHC1 interaction confers Ag specificity, whereas the pMHC1/CD8 interaction mediates enhanced sensitivity to Ag. Striking biophysical differences exist between the TCR/pMHC1 and pMHC1/CD8 interactions; indeed, the pMHC1/CD8 interaction can be >100-fold weaker than the cognate TCR/pMHC1 interaction. In this study, we show that increasing the strength of the pMHC1/CD8 interaction by ∼15-fold results in nonspecific, cognate Ag-independent pMHC1 tetramer binding at the cell surface. Furthermore, pMHC1 molecules with superenhanced affinity for CD8 activate CTLs in the absence of a specific TCR/pMHC1 interaction to elicit a full range of effector functions, including cytokine/chemokine release, degranulation and proliferation. Thus, the low solution binding affinity of the pMHC1/CD8 interaction is essential for the maintenance of CTL Ag specificity. The Journal of Immunology, 2010, 184: 000–000.

C

D8⁺ CTLs recognize antigenic determinants in the form of short peptides derived from endogenous proteins bound to MHC class I (MHC1) 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 (pMHC1), 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
characteristics of the pMHC/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 two 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 26-35) (24, 25). In addition, the following non-A2-restricted CTL clones were used: 1) the HLA A*0801-restricted CTL clone c23, specific for the HIV-1 Tat-derived epitope ITKGLGISYGR (residues 38-48) (26); 2) the HLA B*0702-restricted CTL clone KD4, specific for the EBV EBNA3A-derived epitope PPPFIRRL (residues 37-39); and 3) the HLA A*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 U/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 transduced cells were maintained with puBuLLet-human CD8a (30, 31) into 293T cells using vesicular stomatitis virus-pseudotyped Moloney murine leukemia virus particles. The 293T-CD8a cells were manufactured by introducing puBuLLet-human CD8a (30, 31) into 293T cells using vesicular stomatitis virus-pseudotyped Moloney murine leukemia virus particles. The 293T-CD8a 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). Hmy2 C1R B (Cir) cells expressing full-length A2 and variants thereof were generated as described previously (21).

pMHC1 tetramers

Tetrameric complexes of wild-type pMHC1 molecules and mutants thereof were produced, stored and used as described previously (9, 21). The following A2-restricted peptide tetramers used to refold the pMHC1 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); NLVPMATV (CMV pp65, residues 495-503); ELAGIGILTV (Melan-A, residues 26-35) and ILAKFLHWL (hTERT, residues 540-548). Tetrameric or multimeric pMHC1 reagents were constructed by the addition of streptavidin conjugated to PE, quantum dot 605 or quantum dot 800 (Life Technologies) and 100 µg/ml streptavidin (Life Technologies) at concentrations indicated for 20–30 min at 37°C. Cells were subsequently stained with 2.5 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 FACS-Calibur 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 10^6 003 CTLs per well were resuspended in a 96-well round-bottomed plate with various concentrations of the indicated PE-conjugated tetramers (A2 SLYNTVATL, A2/Kb SLYNTVATL, A2 LLFGYPVYV, or A2/Kb LLFGYPVYV) diluted in 40 µl RPMI 1640 containing 2% FCS plus penicillin, streptomycin, and glutamine as described previously (22) 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 R-PA-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 FACS-Calibur flow cytometer and analyzed with CellQuest software (BD Biosciences).

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

CTLs were incubated with CIR A2 cells, CIR A2/Kb 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 FACS-Calibur flow cytometer and analyzed with CBA software (BD Biosciences). For tetramer-based ELISPOT assays, 2 × 10^5 CTL ± pMHC1 tetramer at 1 µg/ml were applied to duplicate wells of PVDF-backed plates (Millipore, Bedford, MA) precoated with IFN-γ capture Ab 1-D1K (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/T225A 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 CIR A2 cells, CIR A2/Kb cells or medium alone at different E:T ratios; alternatively, CTLs were incubated with various pMHC1 tetramers. Both FITC-conjugated anti-CD107a (clone H4A3; BD Biosciences) and 0.7 µM molsenin (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 FACS-Calibur flow cytometer and analyzed with FlowJo software (Tree Star).

CTL priming assay

Transfected CIR cells were pulsed with 1 µM ELAGIGILTV (Melan-A2b,35) peptide for 90 min, irradiated, and washed once in RPMI 1640 medium. Pulsed, irradiated CIR 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-A2b,35 were quantified on day 10 with wild-type A2 ELAGIGILTV tetramer.

Results

Generation of MHC1 molecules with superenhanced CD8 binding affinity

Tetrameric fusion molecules comprising the α1/α2 peptide binding platform of A2 and the α3 domain of H2-Kb (A2/Kb from this point forward) enable the monitoring of CD8+ T cell responses in A2 transgenic mice (33). This reflects a requirement for the murine MHC1 α3 domain to engage murine CD8 (11), thus enabling A2/Kb reagents to stain murine CTL with lower affinity TCR/pMHC1 interactions (so-called “low avidity” CTLs) (22). The A2/Kb H chain folded with human β2m interacts strongly with human CD8 (Kb ~10 µM, compared with A2 that binds to CD8 with a Kd ~150 µM) but exhibits unaltered A2-restricted TCR binding properties (9, 22). Thus, fusing the α3 domain with A2 α1/α2 domains increases
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/Kb 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 Gag77–85), each expressing a different TCR (19, 22, 23) (Fig. 1A). Noncognate A2 LLFGYPVVY (HILV-1 Tax11–19) tetramers failed to stain any of these in vitro expanded CTL populations to any notable extent. However, A2/Kb SLYNTVATL and A2/Kb LLFGYPVVY tetramers stained the non-cognate CD8+ cell population in the 868 CTL line (Fig. 1A). To examine this effect in more detail, we used A2 and A2/Kb 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 LLFGYPVVY tetramers (Fig. 1B). In contrast, both the A2/Kb SLYNTVATL and A2/Kb LLFGYPVVY tetramers stained >85% of CD8+ cells in PBMCs (Fig. 1B); similar data were obtained with A2/Kb GLCTLVAL (EBV BMLF1259–267) and A2/Kb NLVPMVATV (CMV pp65549–553) 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/Kb tetramers bind the majority of CTLs in peripheral blood

Noncognate A2/Kb tetramers were observed to bind ~80% of the CD8α population in peripheral blood (Fig. 1B). 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/Kb 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. 2A). The majority of γδ-TCR+ (~93.6%) and NK cells (~77%) failed to stain with the A2/Kb ILAKFLHWL (hTERT540–548) tetramer and no significant binding was observed with the corresponding A2 tetramer (Fig. 2B). However, the vast majority of αβ-TCR+/CD8+ cells within the lymphocyte population stained nonspecifically with the A2/Kb ILAKFLHWL tetramer (Fig. 2C).

We hypothesized that most γδ-TCR+ cells and NK cells might fail to bind A2/Kb 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. 3A) to examine the ability of A2/Kb 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/Kb tetramers bound to most (74.3%) of the CD8αα+ 293 T cell transfectants (Fig. 3A,3B); no binding was observed in the absence of CD8αα surface expression (Fig. 3A). Thus, A2/Kb tetramers are capable of binding to cell surface CD8αα.

Why do A2/Kb tetramers bind predominantly to the CTL population in peripheral blood and not to other cells that express CD8?

Fig. 3B shows that A2/Kb tetramer staining is directly proportional to the level of CD8αα expression, such that only cells with a higher level

![FIGURE 2.](http://www.jimmunol.org/)

A2/Kb tetramers bind the majority of CTLs in peripheral blood. A. 2.5 × 10⁵ 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 re-suspended in PBS. B. 2.5 × 10⁵ A2+ PBMCs were stained with 10 μg/ml of the PE-conjugated tetramers A2 ILAKFLHWL or A2/Kb 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 re-suspended in PBS. C. 2.5 × 10⁵ A2+ PBMCs were stained with 10 μg/ml of the PE-conjugated tetramers A2 ILAKFLHWL or A2/Kb 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 re-suspended 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 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/Kb tetramers. A2/Kb 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/Kb tetramer binding at the cell surface (Figs. 1–3) could activate human CTLs. Initially, we studied the A2-restricted SLYNTV ATL-specific CTL clone 003 (23). Consistent with our findings previously stated, both A2 SLYNTVATL and A2/Kb SLYNTVATL tetramers stained 003 CTLs efficiently, as did the noncognate A2/Kb 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/Kb SLYNTVATL and A2/Kb 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/Kb 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/Kb GLCTLV AML tetramer, in this case folded around the GLCTLV AML peptide, induced degranulation in 15% of 003 CTLs at a concentration of 5 μg/ml (Fig. 4F); the cognate A2 SLYNTVATL and A2/Kb SLYNTVATL tetramers induced almost 40% degranulation (data not shown). Notably, the cells that degranulated in response to the A2/Kb GLCTLV AML tetramer were contained...
almost exclusively within the tetramer\textsuperscript{high}CD8\textsuperscript{high} population (Fig. 4F). Thus, at least to some extent, the strong interaction between A2/K\textsuperscript{b} and CD8 can bypass the requirement for a specific TCR/pMHCI interaction and nonspecifically activate human CTLs.

Cell surface-expressed A2/K\textsuperscript{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\textsuperscript{b}; stable transfectants expressing similar cell surface MHCI densities were selected as targets for further experiments. Target cells expressing either A2 or A2/K\textsuperscript{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. 5A). Remarkably, however, the A2/K\textsuperscript{b} targets stimulated Mel13, 003, and ILA1 CTLs to produce significant amounts of MIP-1\textbeta in the absence of specific peptide (Fig. 5A). A2/K\textsuperscript{b} targets also elicited substantial levels of TNF\textalpha and IFN-\gamma at titratable E:T ratios (Fig. 5B), induced degranulation (Fig. 5C), and induced significant levels of killing (data not shown) in the absence of specific TCR/pMHCI interactions.

Cell surface-expressed A2/K\textsuperscript{b} primes noncognate CTL expansions

Thymic output in healthy A2\textsuperscript{+} individuals is known to generate a high frequency of na"ive CD8\textsuperscript{+} T cells that can recognize the self-Ag Melan-A\textsubscript{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\textsubscript{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.
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-A26–35; 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/Kb C1R targets, respectively (Fig. 6). Exposure to A2/Kb 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/Kb-mediated CTL activation and tetramer staining are not dependent on TCR expression

In earlier experiments, we observed that A2/Kb tetramers bound to the majority of ab-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 A2ab PBMCs. As previously, the A2/Kb ILAKFLHWL tetramer bound nonspecifically to the majority of CD8+ cells (Fig. 7A). Furthermore, A2/Kb tetramer binding favored CD8high 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/Kb 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/Kb, 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/Kb activated CTL clones regardless of restriction element (Fig. 7B).

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/Kb 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 binding.
activate CTL clones. Notably, we found that A2/Kb tetramers activated specific expansion of CD8+ cells during the course of the experiment.

CD8 for 20 min on ice, then stained with 10^6 M FITC-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.

[w ithin PBMCs (Figs. 1, 2, 7A). 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/CD8α cocrystals exhibit similar binding orientations (14, 43). Furthermore, these results are consistent with the observation that thymus leukemia Ag, which interacts strongly (Kd ~12 μM) with cell surface CD8α 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/Kb molecules exhibited no peptide specificity requirements whatsoever; indeed, cell surface-expressed A2/Kb 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/Kb to engage multiple CD8 molecules at the cell surface, these results suggest that A2/Kb 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 if 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 pMHC/CD8 interaction is significantly stronger (KD ~30 μM) than the equivalent human interaction (KD ~150 μM) (11), but does not result in noncognate CTL activation. It is therefore likely that a pMHC/CD8 interaction affinity threshold exists for the maintenance of CTL activation specificity. The strength of the murine pMHC/CD8 interaction is 3-fold weaker than the strength of the interaction measured between A2/Kb and human CD8, thereby maintaining of CTL activation specificity. The strength of the murine pMHC/CD8 interaction is essential for the preservation of pMHC ligand binding specificity at the cell surface and its attendant functional repercussions.

Acknowledgments

We thank E. Wang for provision of the allophycocyanin-conjugated mAb specific for human CD56.

Disclosures

The authors have no financial conflicts of interest.

References


