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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 Debet,‡ 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: 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/CD3ζ 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
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) IIA1, specific for the human telomerase reverse transcriptase (HTERT)-derived epitope ILAKFLHWL (clone SK1; BD Biosciences), PerCP-conjugated anti-human CD8 (clone RPA-T8; BD Biosciences, San Jose, CA), FITC-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-human CD107a (clone H4A3; BD Biosciences), and allophycocyanin-conjugated anti-human CD8 (clone BMA 031; Serotec, Oxford, U.K.), FITC-conjugated or PE-conjugated anti-human CD107a (clone H4A3; BD Biosciences) and 0.7 ml/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 CellQuest (BD Biosciences) and FlowJo (Tree Star, Ashland, OR) software.

TCP 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/Kb SLYNTVATL, A2 LLFGPVYYV, or A2/Kb LLFGPVYYV) 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 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 A2Kb 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 (BD 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 × 10^5 CTL ± pMHC 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/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, Straasberg, Germany).

Degranulation assay

Surface CD107a mobilization was used to assess degranulation as described previously (22). Briefly, CTLs were incubated for 4 h at 37°C with either C1R A2 cells, C1R A2Kb cells or medium alone at different E:T ratios; alternatively, CTLs were incubated with various pMHC tetramers. Both FITC-conjugated anti-CD107a (clone HA3A; BD Biosciences) and 0.7 μM/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 pulse with 1 μM ELAGIGILTV (Melan-A26-35) 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-A26-35 were quantified on day 10 with wild-type A2 ELAGIGILTV tetramer.

Results

Generation of MHC 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^d (A2/K^d from this point forward) enable the monitoring of CD8⁺ T cell responses in A2 transgenic mice (33). This reflects a requirement for the murine MHC α3 domain to engage murine CD8 (11), thus enabling A2/K^d reagents to stain murine CTL with lower affinity TCR/pMHC interactions (so-called “low avidity” CTLs) (22). The A2/K^d H chain folded with human β2m interacts strongly with human CD8 (Kd ~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 murine 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 LLFGYPVVV (HILV-1 Tax11-19) tetramers failed to stain any of these in vitro expanded CTL populations to any notable extent. However, A2/Kb LLFGYPVVV tetramers stained all SLYNTVATL-specific CTLs; in addition, both the A2/Kb SLYNTVATL and A2/Kb LLFGYPVVV 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 LLFGYPVVV tetramers (Fig. 1B). In contrast, both the A2/Kb SLYNTVATL and A2/Kb LLFGYPVVV tetramers stained >85% of CD8+ cells in PBMCs (Fig. 1B); similar data were obtained with A2/Kb NLVPMA TV (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 by ~15-fold without affecting the TCR/pMHCI interaction.

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 x 10^6 cells), all specific for HIV-1 p17 Gag77-85, were stained with 1 μg of the PE-conjugated tetramers A2 SLYNTVATL, A2/Kb SLYNTVATL, A2 LLFGYPVVV, or A2/Kb LLFGYPVVV 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 x 10^6 PBMCs were suspended in 250 μl FACS buffer (2% FCS/PBS), then stained with 1 μg of the PE-conjugated tetramers A2 SLYNTVATL, A2/Kb SLYNTVATL, A2 LLFGYPVVV, or A2/Kb LLFGYPVVV 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.

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interaction is increased by \(\sim 15\) -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 \(\sim 80\%\) of the CD8\(^a\) population in peripheral blood (Fig. 1B). Although CD8\(^a\) 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\(^a\) cells that stain with A2/K\(^b\) tetramers. Staining of fresh ex vivo PBMCs isolated from healthy A2\(^+\) donors revealed that CD8\(^a\) 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 (hTERT\(_{540-548}\)) tetramer and no significant binding was observed with the corresponding A2 tetramer (Fig. 2B). However, the vast majority of \(\alpha\beta\)-TCR\(^+\)/CD8\(^a\) 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\(^a\) homo-dimer rather than the CD8\(^a\) heterodimer, which is expressed on the surface of CTLs. Thus, we generated a 293T cell line that expressed CD8\(^a\) (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\(^a\) \(\gamma\delta\)-TCR\(^+\) 293 T cell transfectants (Fig. 3A, 3B); no binding was observed in the absence of CD8\(^a\) surface expression (Fig. 3A). Thus, A2/K\(^b\) tetramers are capable of binding to cell surface CD8\(^a\).

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\(^a\) expression, such that only cells with a higher level...
of CD8\textsuperscript{a} expression stain with this reagent. Examination of PBMCs from healthy donors revealed that CD8\textsuperscript{+} cells express high levels of CD8, whereas NK and \textgamma\textdelta-TCR\textsuperscript{+} 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 CD8\textsuperscript{a} or CD8\textsuperscript{b}. 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\textsuperscript{b} tetramers.

**A2/K\textsuperscript{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 (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\textsuperscript{b} SLYNTVATL tetramers stained 003 CTLs efficiently, as did the noncognate A2/K\textsuperscript{b} LLFGYPVV tetramer; no staining was observed with the A2 LLFGYPVV 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 LLFGYPVV tetramer (Fig. 4B). In contrast, however, both the A2/K\textsuperscript{b} SLYNTVATL and A2/K\textsuperscript{b} LLFGYPVV 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-\gamma, and MIP-1\textbeta (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\textsuperscript{b} tetramers was less efficient than that induced by tetramers bearing the agonist peptide (Fig. 4D–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 CD107\textalpha mobilized on to the cell surface (32). The noncognate A2/K\textsuperscript{b} tetramer, in this case folded around the GLCTLVAML peptide, induced degranulation in 15% of 003 CTLs at a concentration of 5 \mu g/ml (Fig. 4F); the cognate A2 SLYNTVATL and A2/K\textsuperscript{b} SLYNTVATL tetramers induced almost 40% degranulation (data not shown). Notably, the cells that degranulated in response to the A2/K\textsuperscript{b} GLCTLVAML tetramer were contained...
almost exclusively within the tetramerhighCD8high population (Fig. 4F). Thus, at least to some extent, the strong interaction between A2/Kb and CD8 can bypass the requirement for a specific TCR/pMHCI interaction and nonspecifically activate human CTLs.

Cell surface-expressed A2/Kb 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/Kb; stable transfectants expressing similar cell surface MHCI densities were selected as targets for further experiments. Target cells expressing either A2 or A2/Kb 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/Kb targets stimulated Mel13, 003, and ILA1 CTLs to produce significant amounts of MIP-1β in the absence of specific peptide (Fig. 5A). A2/Kb targets also elicited substantial levels of TNFα and IFN-γ 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/Kb 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-A26–35 (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-A26–35 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 \( \alpha \beta \)-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/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 \( \sim 15 \)-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 \( \sim 15 \)-fold resulted in the total loss of pMHCI

![Figure 5](image_url)

**FIGURE 5.** Cell surface-expressed A2/Kb activates CTLs in the absence of cognate Ag. A. 2.5 \( \times \) 10⁴ Mel13, 003, or ILA1 CTLs were incubated for 12 h at 37°C with 10⁴ C1R cells stably transfected to express equal levels of either A2 or A2/Kb 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 \( \times \) 10⁴ Mel13 CTLs were incubated for 12 h at 37°C with 10⁴ C1R cells stably transfected to express either A2 or A2/Kb 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/Kb on the cell surface. For A–C, C1R cells were not previously pulsed with peptide.

![Figure 6](image_url)

**FIGURE 6.** Cell surface-expressed A2/Kb primes nonspecific expansion of CD8+ cells. 10⁶ A2+ PBMCs were incubated with 2 \( \times \) 10⁵ irradiated A2 D227K/T228A, A2, or A2/Kb C1R cells that had previously been pulsed with 1 \( \mu \)M ELAGIGILTV (Melan-A26–35) 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.
tetramer binding specificity. Thus, irrespective of restriction element and the presented peptide, A2/Kb 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/Kb 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/CD8 specific binding. Thus, irrespective of restriction element and the presented peptide, A2/Kb 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). 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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/Kb molecules. This finding led us to examine the effects of cell-surface presented Ag. Strikingly, exposure of PBMCs to C1R target cells bearing A2/Kb molecules caused a general non-specific expansion of CD8+ cells during the course of the experiment (Fig. 6). Furthermore, A2/Kb+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, 7B). 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 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 magnitude of pMHCI/CD8 interaction is significantly stronger (Kd ~ 30 μM) than the equivalent human interaction (Kd ~ 150 μM) (11), but does not result in noncoagulate 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 human pMHCI/CD8 interaction affinity threshold exists for the maintenance of CTL activation specificity. 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) non-specific 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

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References


