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High Avidity Antigen-Specific CTL Identified by CD8-Independent Tetramer Staining¹

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Tetrameric MHC/peptide complexes are important tools for enumerating, phenotyping, and rapidly cloning Ag-specific T cells. It remains however unclear whether they can reliably distinguish between high and low avidity T cell clones. In this report, tetramers with mutated CD8 binding site selectively stain higher avidity human and murine CTL capable of recognizing physiological levels of Ag. Furthermore, we demonstrate that CD8 binding significantly enhances the avidity as well as the stability of interactions between CTL and cognate tetramers. The use of CD8-null tetramers to identify high avidity CTL provides a tool to compare vaccination strategies for their ability to enhance the frequency of high avidity CTL. Using this technique, we show that DNA priming and vaccinia boosting of HHD A2 transgenic mice fail to selectively expand large numbers of high avidity NY-ESO-1_{157–165}-specific CTL, possibly due to the large amounts of antigenic peptide delivered by the vaccinia virus. Furthermore, development of a protocol for rapid identification of high avidity human and murine T cells using tetramers with impaired CD8 binding provides an opportunity not only to monitor expansion of high avidity T cell responses *ex vivo*, but also to sort high avidity CTL clones for adoptive T cell transfer therapy. *The Journal of Immunology*, 2003, 171: 5116–5123.

T cells with high functional avidity can efficiently recognize viral (1) and tumor Ags (2, 3), and contain virus infection (4) as well as tumor outgrowth (2, 5). Protocols to identify high avidity T cells would provide an opportunity to analyze in *ex vivo* assays the interclonal T cell competition during T cell expansion and to sort high avidity T cell clones to be used in adoptive T cell transfer therapy (6). Several factors can influence the avidity of interactions between TCR and MHC class I/peptide complexes, including the density and stability of TCR/MHC class I peptide complexes (7–9), the colocalization of TCR and CD8 in the immune synapse (7, 10–13), and the binding of CD8 molecules (14). Initial reports suggested that the intensity of staining by tetrameric soluble MHC/peptide complexes (tetramers) could be used as a parameter to identify high avidity T cells (15–18), and more recently it has been shown that stability of tetramer binding to T cell clones could be used to assess the affinity of TCR recognition (19, 20). Brightness of tetramer staining, however, can be influenced by several factors, including tetramer internalization (19, 21, 22), whereas tetramer decay experiments require tetramer half-life measurements that cannot be easily conducted to sort high avidity T cell populations.

During Ag recognition, peptide/MHC (pMHC)³ complexes engage both TCR and its coreceptors, CD8 and CD4 molecules. Although CD4 molecules do not enhance the stability of pMHC-TCR interactions (23, 24), the effects of CD8 remain controversial in surface plasmon resonance (SPR) studies (14, 23, 25, 26). Structural and kinetic analysis of CD8 binding to MHC class I molecules has clarified the molecular motifs involved (26, 27). Affinity of class I/CD8 binding is relatively weak ($\sim 130 \mu\text{M}$), as compared with the affinity of TCR binding to pMHC complexes ($\sim 1\text{--}10 \mu\text{M}$) (26, 28–30), ensuring that CD8⁺ T lymphocytes recognize specifically target cells expressing the cognate pMHC class I complexes. Nonetheless, class I tetramer staining has been shown to be blocked by certain anti-CD8 Abs (31–33). Although this suggests a role of CD8 in stabilizing tetramer binding to CTL, the possibility that anti-CD8 Abs may prevent TCR binding to pMHC via steric hindrance cannot be ruled out. Consistent with the latter possibility, we have observed that anti-CD8 Abs can prevent binding of CD8-null tetramers and block the activation of CTL by APCs that do not allow a pMHC class I/CD8 interaction.⁴

A previous report described qualitative differences between human CTL sorted by wild-type tetramers vs tetramers containing a mutation at position 245 (34), without addressing the relationship between T cell peptide sensitivity and tetramer staining. Because a single amino acid substitution at position 245 in the $\alpha 3$ CD8 binding loop of A2 reduces, without completely abolishing, binding to CD8 molecules (35), it remains to be established whether CD8 binding to class I tetramers is an absolute requirement for staining all CD8⁺ T cells with class I tetramers and whether tetramers lacking CD8 binding site can specifically identify high avidity

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³ Abbreviations used in this paper: pMHC, peptide/MHC; SPR, surface plasmon resonance; rVV, recombinant vaccinia virus.

⁴ L. Wooldridge, S. L. Hutchinson, E. M. Choi, A. Lissina, E. Jones, F. Mirza, P. R. Dunbar, D. A. Price, V. Cerundolo, and A. K. Sewell. Anti-CD8 Abs can inhibit or enhance peptide-MHC class-I (pMHCI) multimer binding: this is paralleled by their effects on cytotoxic T lymphocyte activation and occurs in the absence of an interaction between pMHCI and CD8 on the cell surface. *Submitted for publication*.

CTL. Because murine CD8 molecules fail to bind to the $\alpha 3$ domain of human class I molecules (29), tetramer staining of A2-restricted responses in A2 transgenic mice provides a model to address whether high avidity CTL can be identified by A2 tetramers (i.e., tetramers lacking murine CD8 binding site). In contrast, A2K^b tetramers (i.e., chimeric human murine tetramers containing $\alpha 1/\alpha 2$ domain from A2 molecules and $\alpha 3$ domain from K^b molecules), engineered to contain the murine CD8 binding site, should be able to stain both high and low avidity T cells. Consistent with this reasoning, we have previously demonstrated that chimeric A2K^b tetramers detect a higher frequency of Ag-specific A2-restricted CTL than A2 tetramers in A2 transgenic mice (36).

To address whether high avidity T cells can be specifically stained by CD8-null tetramers, we have analyzed the immune response specific to the cancer-testis Ag NY-ESO-1₁₅₇₋₁₆₅ A2-restricted epitope in β_2 -microglobulin^{-/-} A2-D^{b+/+} D^{b-/-} (HHD A2) transgenic mice (37) and demonstrated that A2 NY-ESO-1₁₅₇₋₁₆₅ tetramers stain high but not low avidity NY-ESO-1₁₅₇₋₁₆₅-specific CTL. Staining by A2K^b tetramers failed to distinguish between low and high avidity NY-ESO-1₁₅₇₋₁₆₅-specific CTL lines. Finally, we have extended these observations to the staining of human tumor and virus-specific CTL by engineering tetramers lacking the human CD8 binding site and demonstrating their ability to stain specifically human CTL clones and lines and to identify high avidity CTL.

Materials and Methods

Tetramer synthesis

A2K^b tetramers containing mutations at residues 226 and 227, mutant 226/227AK A2K^b monomer construct, was made by PCR-based site-directed mutagenesis of the wild-type A2K^b template. All MHC class I tetramers were synthesized as previously described (36). Mutant 226/227AK A2K^b and 227/228KA A2 monomers were freshly conjugated before use to avoid issues associated with their relative instability (38).

Murine CTL lines

The A2 transgenic HHD A2 mice (39) were primed by injecting i.m. 50 μ g of the plasmid DNA pSG₂ encoding the full-length NY-ESO-1. Ten days after DNA priming, mice were boosted by i.v. injection of recombinant vaccinia virus (rVV) expressing the minigene NY-ESO-1₁₅₇₋₁₆₅ containing a Val at position 165 (40). Seven days after vaccinia boosting, mice were killed and splenocytes stimulated *in vitro* in the presence of 10 μ M NY-ESO-1₁₅₇₋₁₆₅ peptide in medium containing 100 U/ml IL-2 (PeproTech, Rocky Hill, NJ). Murine CTL lines were generated by weekly peptide stimulation of splenocytes, and tetramer sorting (36).

Human CTL clones

Melan-A₂₆₋₃₅-specific CTL clone 1D4 was generated from a melanoma patient, and tyrosinase₃₆₉₋₃₇₇-specific clones A and B (3G10 and 3F7) from a healthy donor by tetramer sorting, as previously described (41). HIV-1 p17 Gag-specific clones C, D, and E (5C3, 5D3, and 5D8) were sorted from a healthy donor using tetramer, whereas clone F (clone 003) was derived from an HIV-1 patient by limiting dilution (42).

Flow cytometry

T cells were stained with 12.5 ng/ μ l tetramer for 25 min at 37°C. Ab costaining was performed on ice for 20 min, and in blocking experiments, anti-CD8 Ab CT-CD8a was added before tetramer staining (43). Anti-murine CD8 Abs used in this study were CT-CD8a, CT-CD8b, 5H10 (Caltag Laboratories, Burlingame, CA), and anti-human CD8 used was HIT8a (BD PharMingen, San Diego, CA). For phenotype studies, murine CTL lines were stained with Abs against CD2, CD3, CD8 α , CD8 β , CD25, CD28, CD38, CD43, CD44, CD45, CD62L, CD69, CD95L, CTLA-4, NKG2A/C/E, PSGL-1, and TCR $\alpha\beta$ (Caltag Laboratories and BD PharMingen). All analysis was performed on a FACSCaliber (BD Biosciences, Mountain View, CA) using CellQuest software, gating on propidium iodide low live lymphocyte populations.

Tetramer decay assay

T cells were stained with 12.5 ng/ μ l tetramer in 0.1% azide on ice for 20 min and washed extensively. An excess of 100 ng/ μ l competing unlabelled

cognate tetramer or unlabelled anti-HLA-A2 Ab (clone BB7.2; Serotec, Kidlington, U.K.) were added to the cells at room temperature and analyzed at specific time points.

Chromium release assay

Target cells were labeled by incubating 2×10^6 cells in 50 μ l of RPMI 1640 (10% FCS) with 100 μ Ci ⁵¹Cr for 30 min. These were then washed extensively before coincubating with day 7 effector cells. Jurkat cells transfected with A2K^b were used as targets at an E:T ratio of 5:1. Total release was determined in the presence of 5% Triton X-100. Supernatants were collected at 4 h, and radioactivity measured by gamma counting. The percentage of specific lysis was calculated as: $100 \times (\text{experimental} - \text{spontaneous release}) / (\text{total} - \text{spontaneous release})$. Each value was calculated as the average of triplicates and shown with SEM.

IFN- γ secretion assay

IFN- γ secretion by CTL was monitored using MACS Mouse IFN- γ Secretion Assay Detection kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. A2 transfected 221 cells were either peptide-pulsed or infected with rVV for 2 h, before coincubating with CTL at an E:T ratio of 1:5 to 1:10 for another 3 h. Cells were then stained with IFN- γ capture and IFN- γ detection Abs for flow cytometry analysis.

IFN- γ ELISPOT assay

A total of 1×10^4 C1R-A2 target cells (29) and 7×10^2 CTL were coincubated for 4 h with or without relevant peptide at specified concentrations. Spots were counted using an ELISPOT Reader System ELR02 (Autoimmun Diagnostika, Strassburg, Germany). SD of the mean of two duplicate assays is shown.

Macrophage inflammatory protein (MIP)-1 β ELISA

C1R-A2 and .45 target cells were pulsed with peptide for 1 h, before incubating with CTL for 4 h at an E:T ratio of 1:1. MIP-1 β concentration in the supernatant was quantified by DuoSet ELISA development kit (R&D Systems, Minneapolis, MN), according to the manufacturer's protocol. Results are presented as percentages of maximum cytokine release by each clone.

SPR studies

BIAcore molecular interaction analysis was performed using a BIAcore 3000TM machine and a CM-5 sensor chip modified with streptavidin using standard amine coupling, as previously described (29). pMHC complexes were immobilized on the surface of individual cells to a concentration of ~ 1000 response units. CD8 $\alpha\alpha$ homodimers and TCR were prepared as previously described (29).

Results

Differential requirement of CTL lines for CD8 binding in tetramer staining

To assess whether staining with tetramers lacking the CD8 binding site could be used to identify high avidity CTL, NY-ESO-1₁₅₇₋₁₆₅-specific T cell lines were generated from A2 transgenic mice immunized with plasmid DNA and vaccinia virus encoding the NY-ESO-1₁₅₇₋₁₆₅ epitope (36). All the T cell lines were phenotypically similar (see *Materials and Methods*) and had similar expression levels of CD8 and TCR (data not shown). Staining of four cell lines with A2 and A2K^b tetramers loaded with the NY-ESO-1₁₅₇₋₁₆₅ peptide revealed two distinct patterns of staining; whereas the line H5 was efficiently stained by both A2 and A2K^b tetramers, a large proportion of the H2 line was stained by the A2K^b tetramers but not by the A2 tetramers (Fig. 1). A similar dichotomy was observed with two additional T cell lines (F6 and F11), which were enriched for NY-ESO-1₁₅₇₋₁₆₅-specific CTL by A2K^b tetramer guided sorting. Because SPR measurements demonstrated that soluble murine CD8 $\alpha\alpha$ homodimers fail to bind to immobilized A2 monomers (29), staining of murine CTL by A2 tetramers (i.e., staining of CTL lines H5 and F11) can be considered as CD8-independent, whereas staining of the lines H2 and F6 is CD8 dependent. Consistent with this conclusion, the presence of blocking

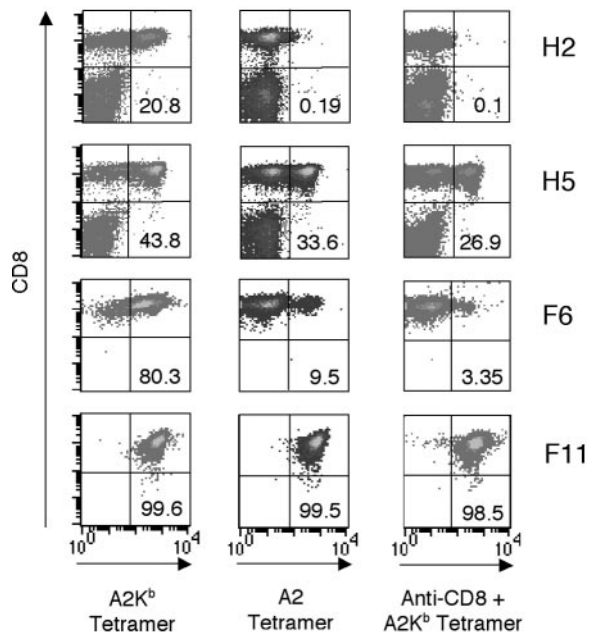


FIGURE 1. CD8-dependent and -independent tetramer staining. Murine NY-ESO-1₁₅₇₋₁₆₅-specific CTL lines were stained using A2K^b (left column) and A2 (middle column) tetramers. Staining with anti-CD8 Abs followed by A2K^b tetramers is shown (right column). The percentages of NY-ESO-1₁₅₇₋₁₆₅-specific CTL are shown in the bottom right quadrant of each panel. Lines F6 and F11 were enriched by A2K^b tetramer guided sorting.

anti-CD8 α Abs abolished binding of A2/K^b tetramers to the CD8 dependent CTL lines H2 and F6 (Fig. 1).

To rule out that lack of staining of the H2 lines by A2 tetramers was due to suboptimal tetramer concentration (Fig. 2A), we compared staining of the H2 and H5 T cell lines by serial dilutions of A2 and A2K^b tetramers. Although the staining intensity of the H5 line by A2 and A2K^b tetramers was above the background at all tetramer concentrations, staining of the H2 line by A2 tetramers was not significantly higher than staining by irrelevant A2 or K^b tetramers (Fig. 2A).

These results demonstrate that the murine class I/CD8 interaction is not critical for the binding of A2K^b tetramers, because some CTL lines (such as the H5 and F11 lines) can be stained by CD8-null tetramers (i.e., A2 tetramers).

Stability of tetramer binding to CTL is enhanced by the presence of CD8 binding to class I molecules

Because the affinity of TCR/pMHC interaction is mainly controlled by its rate of dissociation (44), tetramer decay assays were performed to compare the stability of A2 and A2K^b tetramer binding. Experiments were conducted using the NY-ESO-1₁₅₇₋₁₆₅ CTL lines H5 (Fig. 2B) and H2 (data not shown) upon A2K^b tetramers sorting. The results of these experiments demonstrated that the stability of A2K^b and A2 tetramers bound to high and low avidity CTL lines (see Fig. 3) was significantly different, showing a faster dissociation rate of CD8-null A2 tetramers (Fig. 2B). Similar results were obtained with human CTL clones by comparing the binding of wild-type A2 tetramers vs CD8-null 227/228KA A2 mutant tetramer to HIV-1 p17 Gag₇₇₋₈₅-specific CTL clone (42) (Fig. 2C). Abolishment of CD8 binding increased the off-rate of the tetramers by at least 5-fold.

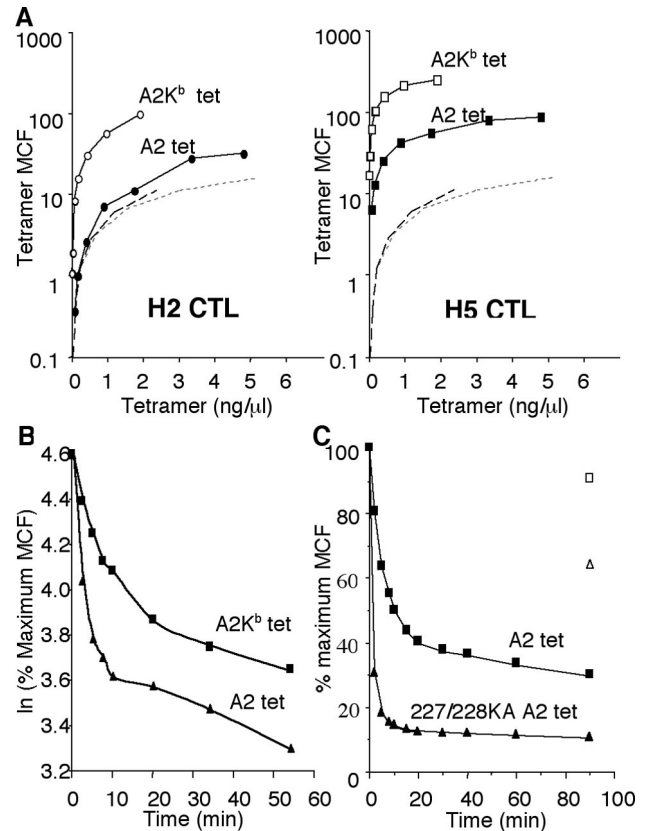


FIGURE 2. CD8 binding stabilizes class I tetramer staining. A, H2 and H5 CTL lines were stained with increasing concentrations of NY-ESO-1₁₅₇₋₁₆₅ A2 (● and ■, respectively) and A2K^b tetramers (○ and □), as well as A2 (light dotted line) and K^b (dark dotted line) tetramers loaded with irrelevant peptides. B, Off-rate measurement of A2 (▲) and A2K^b (■) tetramers from the murine CTL line H5 at room temperature in the presence of excess unlabelled A2K^b tetramers. C, Off-rate measurement of A2 (■) and CD8-null 227/228KA (▲) A2 tetramers binding to the human CTL clone 003 specific for HIV-1 p17 Gag₇₈₋₈₅ in the presence of anti-A2 Ab BB7.2. A2 (□) and CD8-null 227/228 KA (△) A2 tetramers staining of clone 003 in the absence of BB7.2.

CD8-null tetramer staining identifies high avidity murine CTL

To assess whether CTL staining by tetramers lacking CD8 binding site can be used to identify high avidity CTL lines, we assessed the

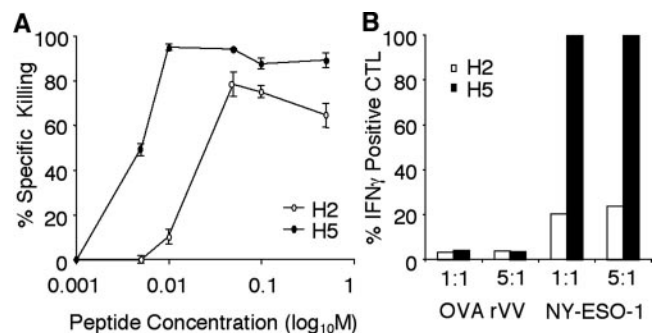


FIGURE 3. Higher functional avidity of CTL lines detectable by CD8 null tetramers. A, NY-ESO-1₁₅₇₋₁₆₅ murine CTL lines H2 and H5 were tested for their ability either to kill peptide-pulsed A2K^b-positive target cells or (B) to release IFN- γ upon coinubation with A2-positive targets infected with rVV encoding the epitope NY-ESO-1₁₅₇₋₁₆₅. Negative controls with irrelevant vaccinia (OVA rVV)-infected cells is shown.

ability of the murine NY-ESO-1₁₅₇₋₁₆₅-specific lines to lyse peptide-pulsed A2K^b transfected Jurkat cells (Fig. 3A). The amount of peptide required to sensitize 50% of target cells for lysis by H5 and F11 CTL lines was ~10 times lower than the amount of peptide required by H2 and F6 CTL lines (Fig. 3A and data not shown). We then tested the ability of H2 and H5 to recognize targets transfected with A2 cDNA. We showed that although the high-affinity H5 CTL line was capable of secreting IFN- γ upon stimulation with target cells infected with rVV encoding the NY-ESO-1₁₅₇₋₁₆₅ minigene, the H2 CTL line secreted significantly lower amounts of IFN- γ (Fig. 3B). Control experiments with target cells pulsed with saturation amount of NY-ESO-1₁₅₇₋₁₆₅ peptide confirmed that the H2 and H5 CTL lines were capable of secreting similar amount of IFN- γ (data not shown).

DNA priming followed by vaccinia boosting expands both high and low avidity CTL

Development of an ex vivo assay to rapidly identify high avidity T cells provides an opportunity to assess whether high avidity CTL can be selectively expanded during vaccination protocols. To answer this question, we measured the percentage of A2 tetramer-positive CTL over the total number of A2K^b tetramer-positive CTL in DNA primed and vaccinia boosted HHD A2 transgenic mice (Fig. 4). Although NY-ESO-1₁₅₆₋₁₆₇-specific CTLs were detectable by ex vivo A2K^b tetramer staining in all vaccinated mice, only 40% of vaccinated mice (11 of 28) had responses detectable by A2 tetramers (data not shown). Analysis of the percentage of NY-ESO-1₁₅₇₋₁₆₅-specific CTL stained by A2 tetramers over A2K^b-positive CTL revealed that in a large proportion of mice this ratio remained unchanged after boosting with rVV (Fig. 4). Because HHD A2 transgenic mice were boosted with vaccinia virus encoding the optimal length NY-ESO-1₁₅₆₋₁₆₇ epitope, it is possible that an excess of antigenic peptide may have prevented the preferential expansion of high avidity NY-ESO-1₁₅₆₋₁₆₇-specific CTL (4).

Generation of CD8 binding site null tetramers capable of staining human CTL clones

We then sought to address whether results generated in A2 transgenic mice could be extended to human CTL. Initial experiments were conducted to assess whether A2K^b tetramers could be used to stain human CTL. SPR measurements confirmed that the substitution of the α 3 domain of A2 molecules with the α 3 domain of K^b molecules did not alter the ability of soluble human TCR to recognize peptide-loaded A2K^b monomers (Fig. 5A). We showed that soluble TCR purified from human NY-ESO-1₁₅₇₋₁₆₅-specific CTL clone bound to A2 and A2K^b molecules loaded with the NY-ESO-1₁₅₇₋₁₆₅ peptide with very similar affinity (Fig. 5A). Although human TCR can efficiently bind to A2K^b monomers, the higher affinity of human CD8 $\alpha\alpha$ homodimer to K^b α 3 domain ($K_d = 5 \mu\text{M}$) prevented the use of A2K^b tetramers to stain human CD8⁺ T cells (Fig. 5, B and C). SPR measurements with soluble human CD8 $\alpha\alpha$ homodimers showed that the affinity of human CD8 $\alpha\alpha$ homodimer binding to A2K^b monomer was ~20 times higher than its affinity for A2 monomer (~100 μM) (Fig. 5B), resulting in nonspecific staining of all human CD8⁺ T cells (Fig. 5C).

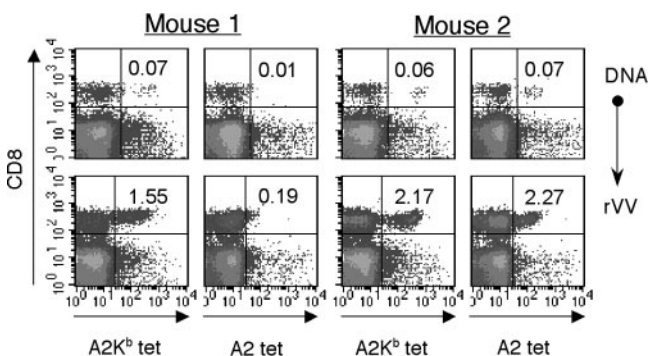


FIGURE 4. Expansion of both high and low avidity T cells by vaccination. Representative staining of immunized HHD mice with A2 and A2K^b tetramers after priming with NY-ESO-1 full-length DNA (DNA) and boosting with vaccinia virus (rVV) encoding the NY-ESO-1₁₅₆₋₁₆₅ minigene. The percentages of tetramer positive CD8⁺ cells are shown in the top right quadrant of each panel.

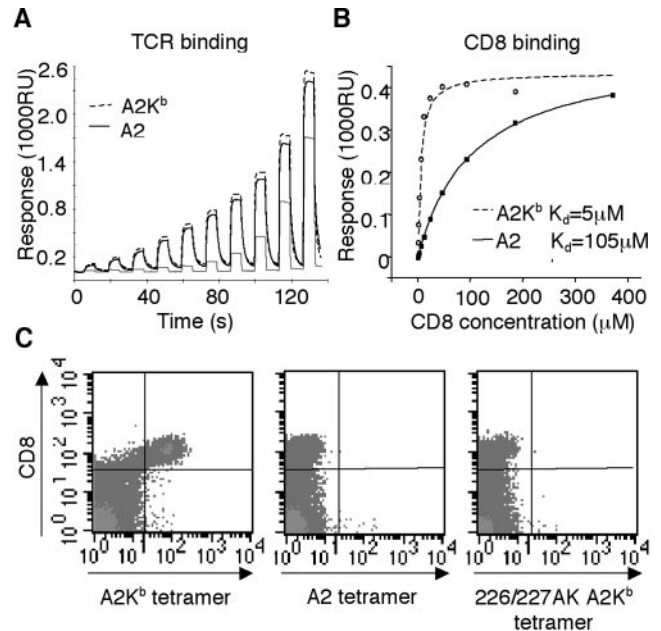


FIGURE 5. Nonspecific staining of human CD8⁺ T cells by A2K^b tetramers. *A*, SPR equilibrium measurement of soluble human NY-ESO-1₁₅₇₋₁₆₅-specific TCR binding to immobilized A2 monomers (solid line), A2K^b monomers (dashed line) loaded with cognate peptide and blank control (gray line). *B*, Binding affinity of human CD8 to A2K^b (dashed line) and A2 (solid line) monomers loaded with the NY-ESO-1₁₅₇₋₁₆₅ peptide. K_d was measured at room temperature and calculated by Scatchard analysis. *C*, Binding of A2K^b, A2, and 226/227AK A2K^b tetramers loaded with the NY-ESO-1₁₅₇₋₁₆₅ peptide to PBL of an A2-positive healthy donor.

Although human TCR can efficiently bind to A2K^b monomers, the higher affinity of human CD8 $\alpha\alpha$ homodimer to K^b α 3 domain ($K_d = 5 \mu\text{M}$) prevented the use of A2K^b tetramers to stain human CD8⁺ T cells (Fig. 5, B and C). SPR measurements with soluble human CD8 $\alpha\alpha$ homodimers showed that the affinity of human CD8 $\alpha\alpha$ homodimer binding to A2K^b monomer was ~20 times higher than its affinity for A2 monomer (~100 μM) (Fig. 5B), resulting in nonspecific staining of all human CD8⁺ T cells (Fig. 5C).

To abolish CD8 binding site on the A2K^b molecules and overcome the high affinity interaction of human CD8 with A2K^b tetramers, we engineered a mutant of A2K^b tetramer in which residues Gln 226 and Asp 227, known to be involved in CD8 binding (45, 46), were mutated to Ala and Lys, respectively. Such Q226A/D227K A2K^b monomers (226/227AK A2K^b) were synthesized and their ability to interact with soluble human CD8 $\alpha\alpha$ analyzed by SPR (Fig. 6A). Although CD8 $\alpha\alpha$ homodimers bind to immobilized A2 monomer with the expected affinity of ~100 μM (26, 29), no binding above the background was detected for 226/227AK A2K^b monomers (Fig. 6A). Correct folding of the 226/227AK A2K^b monomers was confirmed by measuring the binding of anti-A2 and anti- β -2-microglobulin Abs, BB7.1 and BBM.1, and demonstrating a similar binding affinity to A2 and 226/227AK A2K^b monomers (data not shown). Furthermore, we showed that 226/227AK A2K^b tetramers failed to bind nonspecifically to human CD8⁺ T lymphocytes (Fig. 5C), but specifically stained human CTL clones (Fig. 6B).

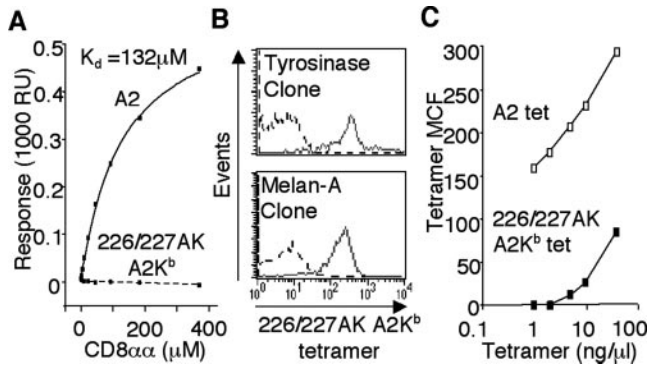


FIGURE 6. Mutation of CD8 binding site in murine K^b α3 domain restores specific staining of human CTL. *A*, SPR equilibrium measurement of soluble human CD8αα homodimer binding to immobilized A2 (solid line) and 226/227AK A2K^b mutant monomers (dashed line) loaded with NY-ESO-1₁₅₇₋₁₆₅ peptide. Binding affinity (K_d) was measured at room temperature and calculated by Scatchard analysis. *B*, Specific staining of tyrosinase₃₆₉₋₃₇₇- and Melan-A₂₆₋₃₅-specific CTL clones by 226/227AK A2K^b tetramers loaded with cognate (solid line) vs noncognate (dashed line) peptide. *C*, Staining of Melan-A₂₆₋₃₅ CTL clone with different concentrations of A2 (□) and 226/227AK A2K^b tetramers (■).

CD8-null tetramers distinguish between high avidity and low avidity human CTL

The ability to stain human CTL clones with CD8-null tetramers provided an opportunity to assess whether results obtained with murine CTL lines could be extended to human CTL, because the

binding affinity of CD8 to MHC is at least 4-fold lower in humans than in mice (29). We first assessed whether CD8 binding to class I tetramers raises the affinity of the interactions between CTL and cognate tetramers. Serial dilution of tetramers showed that A2 tetramers stain CTL clones more efficiently than the 226/227AK A2K^b tetramers (Fig. 6C), confirming that CD8 binding to class I tetramers enhances the binding affinity between TCR and pMHC complexes.

Finally, we assessed whether the correlation between CD8-independent tetramer staining and functional sensitivity of murine CTL could be extended to human CTL. We characterized the staining pattern of several human A2-restricted tumor and virus specific CTL clones, using CD8-binding A2 tetramers and CD8-null A2 tetramers. These experiments were conducted using 226/227AK A2K^b tetramers and the previously described 227/228KA A2 tetramers, bearing mutations at positions 227 and 228, which completely abolish CD8 binding to class I molecules (29).

Experiments conducted with tyrosinase₃₆₉₋₃₇₇-specific CTL clones A and B, demonstrated that tetramer staining of the lower avidity clone A was dependent on CD8 binding, as shown by its lack of staining by CD8-null tetramers. In contrast, the higher avidity clone B was efficiently stained by both wild-type A2 and the CD8-null 227/228KA A2 tetramers (Fig. 7, top panel). Similar results were obtained with the 226/227AK A2K^b tetramers (data not shown).

These results were confirmed using a panel of HIV-1 p17 Gag₇₇₋₈₅-specific CTL clones (Fig. 7, bottom panel). MIP-1β release was measured because this cytokine is a ligand for HIV-1

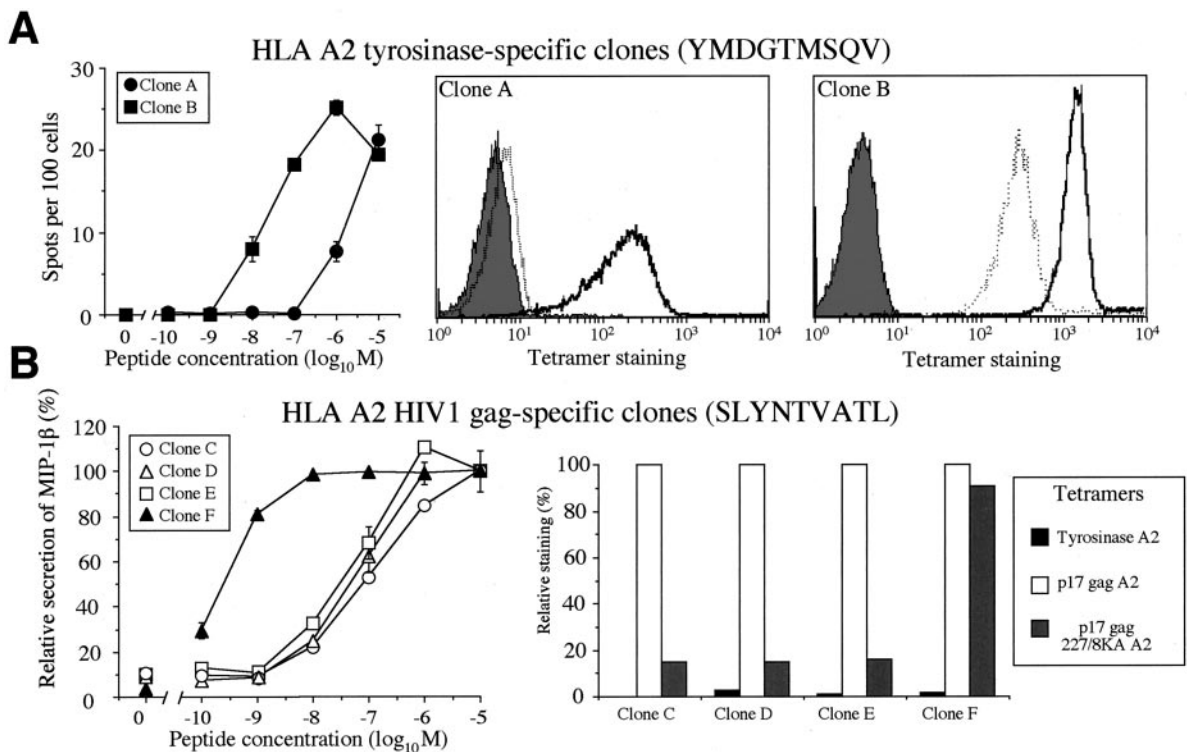


FIGURE 7. CD8-null tetramers distinguish between high and low avidity human CTL. Activation and tetramer staining of tyrosinase₃₆₉₋₃₇₇-specific CTL clones (A) and HIV-1 p17 Gag₇₇₋₈₅-specific CTL clones (B). *A*, A 4 h IFN-γ ELISPOT assay (left panel, top row) with two tyrosinase₃₆₉₋₃₇₇-specific CTL clones. SD of the mean of two duplicate assays is shown. FACS staining (middle and right panels, top row) of these clones. CTLs were stained by A2 (solid line) and 227/228KA-A2 (dashed line) tetramers loaded with the tyrosinase₃₆₉₋₃₇₇ peptide. As a control, cells were stained by A2 tetramers loaded with the HIV-1 p17 Gag₇₇₋₈₅ peptide (shaded line). *B*, MIP-1β secretion (left panel, bottom row) by different HIV-1 p17 Gag₇₇₋₈₅-specific CTL clones upon stimulation by target cells pulsed with serial dilutions of the p17 Gag₇₇₋₈₅ peptide. The ratio of tetramer staining (right panel, bottom row) of HIV-1 p17 Gag-specific CTL clones by A2 (□) and 227/228KA A2 (▣) tetramers loaded with the p17 Gag₇₇₋₈₅ peptide. As a control, cells were stained by A2 tetramers loaded with the (□), cognate peptide 227/228KA A2 tetramers (▣) or A2 tetramer loaded with the tyrosinase₃₆₉₋₃₇₇ peptide (■).

coreceptor CCR5, and has been shown to inhibit HIV-1 infection (47). High avidity clone F is the immunodominant clone from HIV-1-infected patient 003, and has been described extensively elsewhere (42). Lower avidity clones C, D, and E were isolated from an uninfected individual by A2 tetramer guided sorting. Staining of clones C–F with wild-type and CD8-null class I tetramers confirmed that only the high avidity clone F was stained efficiently by the CD8-null tetramer, as compared with the staining of lower affinity CTL clones C, D, and E. Immunodominant A2-restricted CTL specific to other pathogens, including human T cell leukemia virus and Epstein-Barr virus, behave like clone F in that they stain well with both wild-type and CD8-null tetramers (data not shown).

Thus, staining of Ag-specific CTL with CD8-null tetramers permits identification of CTL capable of exerting effector function against cells expressing endogenous Ags.

Discussion

We have previously shown that in A2 transgenic mice, CD8 binding A2K^b tetramers were capable of detecting higher frequency of A2-restricted Ag-specific CTL than A2 tetramers, which are unable to bind to murine CD8 molecules (36, 43). We have now extended these results by showing that CD8-null tetramers selectively identify human and murine CD8-independent CTL with high functional avidity capable of recognizing physiological levels of Ag.

To date, the function of CD8 as a TCR coreceptor has mostly been attributed to its cytoplasmic domains (48) and it is still unclear whether CD8 binding to MHC significantly enhances the stability of interactions between CTL and target cells. Although C terminus of CD8 α is associated with tyrosine kinase p56lck, responsible for TCR phosphorylation during T cell activation, palmitoylation of CD8 β is essential for recruitment into lipid raft (25, 48). Our results, demonstrating the differential capacity of CD8-binding vs CD8-null tetramers to stain CTL, highlight the importance of CD8 ectodomains in facilitating Ag recognition by acting as a bona fide T cell adhesion molecule. The enhanced avidity of CD8-binding over CD8-null tetramers to CTL is clearly demonstrated by tetramer titration experiments, where in some cases at least 5 times as much CD8-null tetramers were required to achieve the same level of intensity as their CD8-binding counterparts (Figs. 2A and 6C). It is possible that the increased overall avidity between target cells and CTL could be accounted for by CD8 functioning as additional binding sites for MHC, independent from TCR. Alternatively, because CD3 δ binds to CD8 (49), induced proximity of CD8 and TCR may result in co-operative binding between CD8 and TCR associating to the same MHC molecules. The latter possibility, however, may prove difficult to be measured by kinetic SPR studies simply using soluble molecules (25, 26).

We have previously shown that a single amino acid substitution at position 245 in the α 3 CD8 binding loop of A2 greatly reduces but does not completely abolish CD8 binding ($K_d = 500 \mu\text{M}$) (35). In contrast, double mutations at position 227 and 228 in the A2 α 3 domain (29) and mutations at 226 and 227 in the K^b α 3 domain (Fig. 6A) reduce the interactions between CD8 and class I molecules to undetectable levels by SPR analysis. Therefore, we decided to avoid the use of class I tetramers bearing mutation A245V to study the contribution of CD8 in stabilizing tetramer binding to T cells (34, 50). Off-rate measurements of wild-type and CD8-null tetramers from murine and human CTL (Fig. 2) clearly demonstrated that CD8 binding to class I molecules significantly influences the avidity of association between TCR and MHC class I complexes and their dissociation rates.

High avidity CTL have been shown to be more efficient in killing tumors *in vitro* and in controlling tumor growth *in vivo* (2, 51).

Similarly, T cells with high peptide sensitivity were more effective in containing viral infection upon adoptive transfer (4). However, it remains unclear whether low avidity T cells are capable of controlling viral infection and tumor proliferation *in vivo*. Furthermore, it remains to be established whether there are differences in the avidity of T cells specific to viral and tumor Ags, because the avidity of the T cell repertoire specific to self tumor Ags may have been shaped by the expression of antigenic proteins by normal cells. Identification of a protocol capable of rapidly identifying high avidity CTL provides an opportunity to assess these issues and to compare different vaccination strategies for their ability to expand high and low avidity CTL responses.

Some of the most successful vaccination protocols for inducing epitope-specific CTL are heterologous “prime-boost” regimens, involving sequential injections of different vectors encoding the same recombinant Ag (43). These vaccination protocols are designed to focus T cell responses on the recombinant Ag, which contains the only T cell epitopes shared by the different delivery vectors, such as DNA and rVV. Although this strategy proves to be very powerful in generating high numbers of CD4⁺ and CD8⁺ T cells specific to a known recombinant gene product, the avidity of T cells expanded by boosting with rVV remains unclear. The results of our experiments demonstrated that boosting of full length NY-ESO-1 DNA primed HHD A2 mice with vaccinia virus encoding the minimal epitope NY-ESO-1_{156–167} failed to specifically expand high avidity CTL (Fig. 4). Several factors are known to play a role in T cell affinity maturation, including tolerance of high affinity repertoire, exhaustion and the size of TCR repertoire (16, 52–56). Our results showing lack of *in vivo* CTL affinity maturation after boosting with vaccinia virus are consistent with previously published data (53–56). In these experimental models, the lack of T cell affinity maturation was accounted for by restricted TCR repertoire in primary responses and clonal senescence, rather than Ag dose. The fact that we were capable of eliciting high and low avidity CTL in HHD A2 mice demonstrates the presence of a broad repertoire of NY-ESO-1_{157–165}-specific T cell response. It is of interest that in A2.1/K^b transgenic mice that express murine class I molecules (57), NY-ESO-1_{157–165}-specific CTL responses were only detectable by A2K^b but not A2 tetramers (36). Indeed, the A2-restricted TCR repertoire in A2.1/K^b mice was found to be narrower than that of HHD A2 mice, probably due to competition during positive selection of the T cell repertoire restricted by the A2 transgene and the endogenous murine class I molecules (58).

Our results suggest that the use of vectors delivering large amounts of optimal peptide epitopes *in vivo* could result in the simultaneous expansion of high and low avidity T cells, rather than in the selective expansion of high avidity T cells. These findings highlight the importance of optimizing protocols to monitor the avidity of expanded T cell populations and to compare in clinical trials T cell avidity with clinical efficacy.

In conclusion, we described a novel protocol to identify high avidity CD8⁺ T cells based on the pattern of staining by CD8 dependent and independent class I tetramers. The lack of CD8/class I interactions destabilizes tetramer binding to T cells and allows selective identification of high avidity CTL. Future vaccination protocols should focus not only on optimizing the expansion of tetramer positive cells, but also on analyzing T cell avidity to ensure that high avidity T cells, capable of recognizing physiological levels of Ags, are preferentially boosted.

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