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*J. Immunol.* 2003;171:5116-5123
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High Avidity Antigen-Specific CTL Identified by CD8-Independent Tetramer Staining

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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-1 187–165-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.

Received for publication May 8, 2003. Accepted for publication September 4, 2003.

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1 This work was funded by the Cancer Research U.K. Programme Grant C399/A2291 and the Cancer Research Institute. E.M.-L.C. was supported by the Medical Research Council, U.K.

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

4 L. Woolridge, S. L. Hutchinson, E. M. Choi, A. Lissina, E. Jones, F. Mirza, P. R. Dunbar, D. A. Price, V. Cerandolo, and A. K. Sewell. Anti-CD8 Abs can inhibit or enhance peptide-MHC class-I (pMHC) multimer binding: this is paralleled by their effects on cytotoxic T lymphocyte activation and occurs in the absence of an interaction between pMHC1 and CD8 on the cell surface. Submitted for publication.
Materials and Methods

Tetramer synthesis

A2Kb tetramers containing mutations at residues 226 and 227, mutant 226/227AK A2Kb monomer construct, was made by PCR-based site-directed mutagenesis of the wild-type A2Kb template. All MHC class I tetramers were synthesized as previously described (36). Mutant 226/227AK A2Kb 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 μg of the plasmid DNA pSG, 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 with 10 μM NY-ESO-1157–165 peptide in the presence of 10 μM NY-ESO-1157–165 peptide in medium containing 10% 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-A2,37–45-specific CTL clone 1D4 was generated from a melanoma patient, and tyrosinase160,237–257-specific clones A and B (3G10 and 3F7) from a melanoma clone. A2-restricted and A2/Kb tetramers were loaded with the NY-ESO-1157–165 peptide, and T cells stained with Abs against CD2, CD3, CD8α, CD8β, CD25, CD28, CD34, CD44, CD45, CD62L, CD69, CD95, CTLA-4, NKG2A/C/E, PSGL-1, and TCRβ (Caltag Laboratories and BD PharMingen, San Diego, CA). All analysis was performed on a FACSCalibur (BD Biosciences, Kidlington, U.K.) using CellQuest software, gating on propidium io-}

Cytometry

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

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 A2Kb 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 A2Kb tetramers, a large proportion of the H2 line was stained by the A2Kb 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 A2Kb tetramer guided sorting. Because SPR measurements demonstrated that soluble murine CD8α 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
anti-CD8 Abs abolished binding of A2/Kb 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 A2Kb tetramers. Although the staining intensity of the H5 line by A2 and A2Kb 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 Kb tetramers (Fig. 2A).

These results demonstrate that the murine class I/CD8 interaction is not critical for the binding of A2Kb tetramers, because some CTL lines (such as the H5 and F11 lines) can be stained by CD8-null tetramers (i.e., A2 tetramers).

FIGURE 1. CD8-dependent and -independent tetramer staining. Murine NY-ESO-1 157–165-specific CTL lines were stained using A2Kb (left column) and A2 (middle column) tetramers. Staining with anti-CD8 Abs followed by A2Kb tetramers is shown (right column). The percentages of NY-ESO-1 157–165-specific CTL are shown in the bottom right quadrant of each panel. Lines F6 and F11 were enriched by A2Kb tetramer guided sorting.

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 A2Kb tetramer binding. Experiments were conducted using the NY-ESO-1 157–165 CTL lines H5 (Fig. 2B) and H2 (data not shown) upon A2Kb tetramers sorting. The results of these experiments demonstrated that the stability of A2Kb 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. 2A).

These results demonstrate that the murine class I/CD8 interaction is not critical for the binding of A2Kb tetramers, because some CTL lines (such as the H5 and F11 lines) can be stained by CD8-null tetramers (i.e., A2 tetramers).

FIGURE 2. CD8 binding stabilizes class I tetramer staining. A, H2 and H5 CTL lines were stained with increasing concentrations of NY-ESO-1 157–165 A2 (● and ■, respectively) and A2Kb tetramers (○ and □), as well as A2 (light dotted line) and Kb (dark dotted line) tetramers loaded with irrelevant peptides. B, Off-rate measurement of A2 (▲) and A2Kb (■) tetramers from the murine CTL line H5 at room temperature in the presence of excess unlabelled A2Kb 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 Gag77–85 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-1 157–165 murine CTL lines H2 and H5 were tested for their ability either to kill peptide-pulsed A2Kb-positive target cells or (B) to release IFN-γ upon coincubation with A2-positive targets infected with rVV encoding the epitope NY-ESO-1 157–165. Negative controls with irrelevant vaccinia (OVA rVV)-infected cells is shown.
ability of the murine NY-ESO-1_{157–165}-specific lines to lyse peptide-pulsed A2K\textsuperscript{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-\(\gamma\) upon stimulation with target cells infected with rVV encoding the NY-ESO-1_{157–165} minigene, the H2 CTL line secreted significantly lower amounts of IFN-\(\gamma\) (Fig. 3B). Control experiments with target cells pulsed with saturation amount of NY-ESO-1_{157–165} peptide confirmed that the H2 and H5 CTL lines were capable of secreting similar amount of IFN-\(\gamma\) (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\textsuperscript{b} tetramer-positive CTL in DNA primed and vaccinia boosted HHD A2 transgenic mice (Fig. 4). Although NY-ESO-1_{156–165}-specific CTLs were detectable by ex vivo A2K\textsuperscript{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-1_{156–165}-specific CTL stained by A2 tetramers over A2K\textsuperscript{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-1_{156–167} epitope, it is possible that an excess of antigenic peptide may have prevented the preferential expansion of high avidity NY-ESO-1_{156–167}-specific CTL (4).

Generation of CD8\textsuperscript{+} 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\textsuperscript{b} tetramers could be used to stain human CTL. SPR measurements confirmed that the substitution of the \(\alpha\)3 domain of A2 molecules with the \(\alpha\)3 domain of K\textsuperscript{b} molecules did not alter the ability of soluble human TCR to recognize peptide-loaded A2K\textsuperscript{b} monomers (Fig. 5A). We showed that soluble TCR purified from human NY-ESO-1_{157–165}-specific CTL clone bound to A2 and A2K\textsuperscript{b} molecules loaded with the NY-ESO-1_{157–165} peptide with very similar affinity (Fig. 5A). Although human TCR can efficiently bind to A2K\textsuperscript{b} monomers, the higher affinity of human CD8\textsuperscript{+} homodimer to K\textsuperscript{b} \(\alpha\)3 domain (\(K_\text{D} = 5 \mu M\)) prevented the use of A2K\textsuperscript{b} tetramers to stain human CD8\textsuperscript{+} T cells (Fig. 5, B and C). SPR measurements with soluble human CD8\textsuperscript{+} homodimers showed that the affinity of human CD8\textsuperscript{+} homodimer binding to A2K\textsuperscript{b} monomer was ~20 times higher than its affinity for A2 monomer (~100 \(\mu M\)) (Fig. 5B), resulting in nonspecific staining of all human CD8\textsuperscript{+} T cells (Fig. 5C).

To abolish CD8 binding site on the A2K\textsuperscript{b} molecules and overcome the high affinity interaction of human CD8 with A2K\textsuperscript{b} tetramers, we engineered a mutant of A2K\textsuperscript{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\textsuperscript{b} monomers (226/227AK A2K\textsuperscript{b}) were synthesized and their ability to interact with soluble human CD8\textsuperscript{+} analyzed by SPR (Fig. 6A). Although CD8\textsuperscript{+} homodimers bind to immobilized A2 monomer with the expected affinity of ~100 \(\mu M\) (26, 29), no binding above the background was detected for 226/227AK A2K\textsuperscript{b} monomers (Fig. 6A). Correct folding of the 226/227AK A2K\textsuperscript{b} monomers was confirmed by measuring the binding of anti-A2 and anti-\(\beta\)-2-microglobulin Abs, BB7.1 and BBM.1, and demonstrating a similar binding affinity to A2 and 226/227AK A2K\textsuperscript{b} monomers (data not shown). Furthermore, we showed that 226/227AK A2K\textsuperscript{b} tetramers failed to bind nonspecifically to human CD8\textsuperscript{+} T lymphocytes (Fig. 5C), but specifically stained human CTL clones (Fig. 6B).
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 avidity 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 A2Kb 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 with 226/227AK A2Kb 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 tyrosinase 369–377-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 A2Kb tetramers (data not shown).

These results were confirmed using a panel of HIV-1 p17 Gag 77–85-specific CTL clones (Fig. 7, bottom panel). MIP-1β release was measured because this cytokine is a ligand for HIV-1
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 A2Kb 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 uncertain whether CD8 binding to MHC significantly enhances the stability of interactions between CTL and target cells. Although C terminus of CD8α is associated with tyrosine kinase p56lck, responsible for TCR phosphorylation during T cell activation, palmitylation of CD8β 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 2C). 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 CD3δ 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 α3 CD8 binding loop of A2 greatly reduces but does not completely abolish CD8 binding (Kd = 500 μM) (35). In contrast, double mutations at position 227 and 228 in the A2 α3 domain (29) and mutations at 226 and 227 in the Kb α3 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-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-157–165-specific T cell response. It is of interest that in A2.1/Kb transgenic mice that express murine class I molecules (57), NY-ESO-157–165-specific CTL responses were only detectable by A2Kb but not A2 tetramers (36). Indeed, the A2-restricted TCR repertoire in A2.1/Kb 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 optional 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.

Acknowledgments

We thank Uzi Gileadi, Dawn Shepherd, Joelle Renneson, and Manuela Herber for technical support.
References


