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# Functional Avidity of Tumor Antigen-Specific CTL Recognition Directly Correlates with the Stability of MHC/Peptide Multimer Binding to TCR<sup>1</sup>

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**Avidity of Ag recognition by tumor-specific T cells is one of the main parameters that determines the potency of a tumor rejection Ag. In this study we show that the relative efficiency of staining of tumor Ag-specific T lymphocytes with the corresponding fluorescent MHC class I/peptide multimeric complexes can considerably vary with staining conditions and does not necessarily correlate with avidity of Ag recognition. Instead, we found a clear correlation between avidity of Ag recognition and the stability of MHC class I/peptide multimeric complexes interaction with TCR as measured in dissociation kinetic experiments. These findings are relevant for both identification and isolation of tumor-reactive CTL. *The Journal of Immunology*, 2002, 168: 1167–1171.**

The use of fluorescent multimeric arrays of peptide/MHC class I complexes (multimers thereafter) allows direct identification and isolation of Ag-specific CD8<sup>+</sup> T cells from heterogeneous populations of lymphocytes. Despite their extensive use in the quantification and characterization of CD8<sup>+</sup> T cell responses (1–3), the molecular bases of the efficiency of flow cytometric staining with multimers and its correlation with T cell effector functions have not been fully elucidated yet. Intuitively, because of the specificity of the interaction between TCR and MHC/peptide complex, the efficiency of multimer staining has been taken as a surrogate measure of TCR affinity for the peptide/MHC complex. Because the latter constitutes one of the main parameters contributing to T cell functional avidity of Ag recognition (operational definition of the overall sensitivity of T cell response to Ag density (4, 5)) it has been assumed that intensity of flow cytometric staining with multimers would directly correlate with the latter. Although initial attempts to apply this concept to the isolation of high-avidity CTL from heterogeneous populations have met with some success (6), discrepancy between multimer binding and functional avidity has also been reported (5, 7–9). In addition, several factors such as experimental staining conditions, interaction with coreceptors, and integrity of lipid rafts have been recently shown to influence multimer binding to CD8<sup>+</sup> T cells (10–12).

Avidity of Ag recognition by tumor-specific CD8<sup>+</sup> T cells strictly correlates with the efficiency of tumor recognition as

shown in several antigenic systems (6, 7) and is indeed one of the main parameters that determines the potency of a tumor rejection Ag (13). With the aim of selectively identifying tumor-reactive CD8<sup>+</sup> T lymphocytes among heterogeneous populations, it is important to determine whether, and under which conditions, staining with fluorescent multimers can provide information on the functional avidity of the populations under study. In this work, we have analyzed the efficiency of multimer staining of tumor Ag-specific T cell clonal populations that recognize the tumor Ag-derived peptide MAGE-A10<sub>254–265</sub> with different functional avidity. We found that the relative efficiency of staining with the corresponding fluorescent MHC class I/peptide multimeric complexes can vary considerably with staining conditions and does not necessarily correlate with avidity of Ag recognition. In contrast, we found a clear correlation between functional avidity and stability of peptide/MHC class I complex interaction with TCR as measured in dissociation kinetic experiments. Similar results were also found in two additional tumor Ag systems.

## Materials and Methods

### Cells

Monoclonal MAGE-A10-specific CD8<sup>+</sup> T cell populations were obtained from melanoma patients and healthy donors as described previously (7). Clones LAU50/15, LAU155/6D1, LAU169/4E8, and healthy donor (HD)<sup>3</sup>795/4D11 were obtained from peptide MAGE-A10<sub>254–262</sub>-stimulated CD8<sup>+</sup> T cells from peripheral blood of melanoma patients LAU50, LAU155, and LAU169, and of donor HD795, respectively. NY-ESO-1 (LAU156/49 and HD007/1F8)- and Melan-A (HD421/4B4 and HD421/1E3)-specific CD8<sup>+</sup> T cell clones were similarly derived from tumor-infiltrating lymph node or peptide-stimulated PBL of the indicated melanoma patient or healthy donor. Polyclonal Melan-A monospecific lines from melanoma patient LAU337 were obtained by ex vivo sorting of Melan-A multimer<sup>+</sup>CD8<sup>+</sup> T cells followed by in vitro stimulation in the presence of PHA and irradiated allogeneic feeder cells as described elsewhere.<sup>4</sup> The melanoma cell line NA8-MEL was kindly provided by Dr. F. Jotereau

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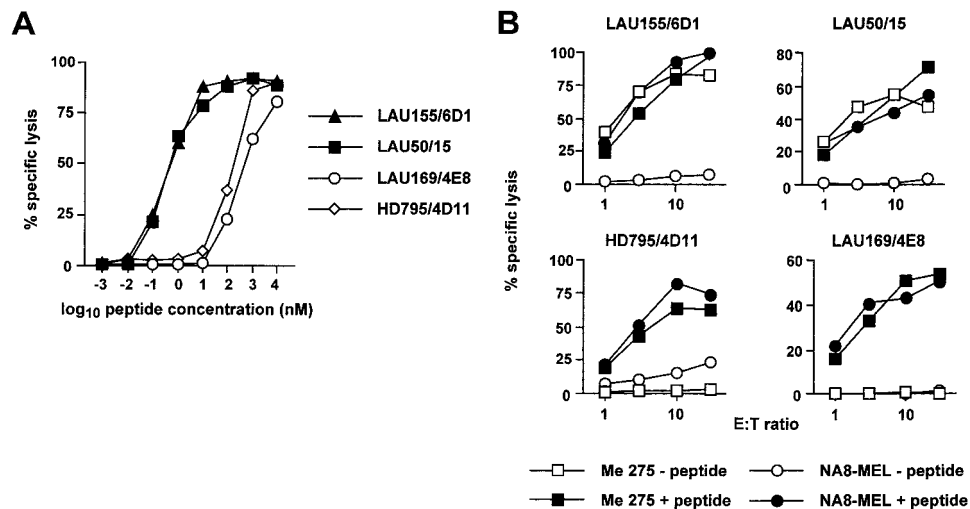
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<sup>3</sup> Abbreviation used in this paper: HD, healthy donor.

<sup>4</sup> D. Valmori, V. Dutoit, V. Schnuriger, A.-L. Quiquerez, M. J. Pittet, P. Guillaume, V. Rubio-Godoy, P. R. Walker, D. Rimoldi, D. Liénard, D. Speiser, J.-C. Cerottini, P. Romero, and P.-Y. Dietrich. Vaccination with a Melan-A peptide selects an oligoclonal T cell population with increased functional avidity and tumor reactivity. *Submitted for publication*.



**FIGURE 1.** Functional avidity of Ag recognition and tumor reactivity of MAGE-A10-specific CTL clones. *A*, Clonal populations were tested for recognition of peptide MAGE-A10<sub>254-262</sub> in a 4-h chromium-release assay using T2 cells as targets at a lymphocyte:target ratio of 10:1 in the presence of graded concentrations of peptide. *B*, Tumor recognition was similarly assessed at the indicated lymphocyte:target cell ratios by using as target cells tumor cell lines Me 275 (HLA-A2<sup>+</sup>MAGE-A10<sup>+</sup>) and NA8-MEL (A2<sup>+</sup>MAGE-A10<sup>-</sup>) in the absence or in the presence of peptide MAGE-A10<sub>254-262</sub> (1 μM).

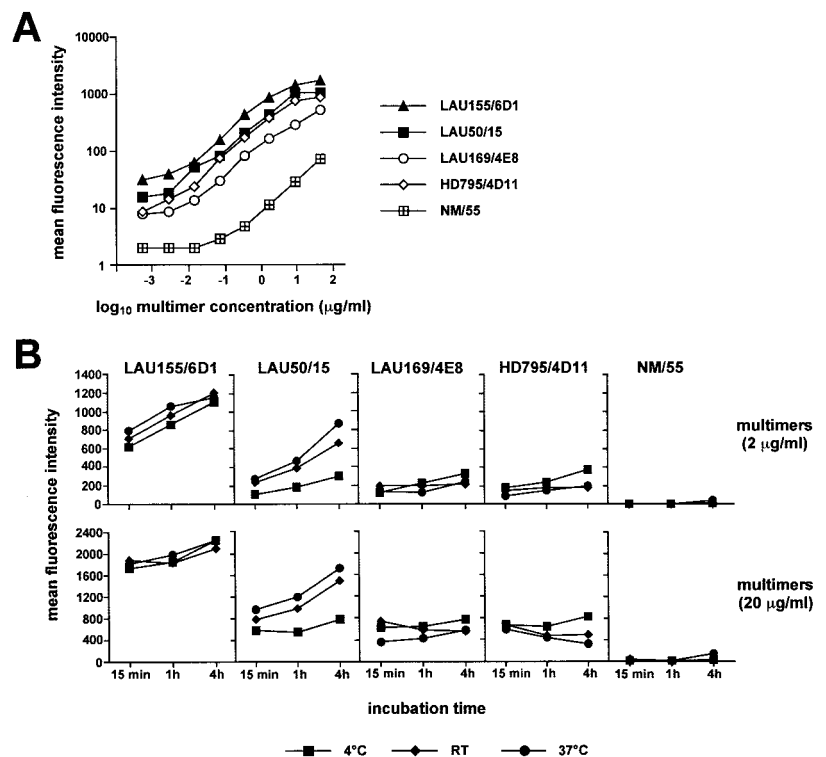
(Institut National de la Santé et de la Recherche Médicale, Unité 463, Nantes, France). The melanoma cell line Me 275 was generated in our laboratory from a surgically excised melanoma metastasis from patient LAU50.

#### Ag recognition assay

Functional avidity of Ag recognition was assessed by chromium-release assay. Briefly, chromium-labeled target T2 cells (1,000 per well) were incubated in the presence of serial dilutions of parental peptide or analogs and effector cells at an E:T cell ratio of 10:1. Tumor recognition was assessed by incubating chromium-labeled target cells (1,000 per well) loaded or not with the indicated peptide (1 μM) with effectors at the indicated ratio. Chromium release was measured in the supernatant after 4 h of incubation at 37°C. The percentage of specific lysis was calculated as 100 × (experimental spontaneous release/total spontaneous release).

#### MHC/peptide multimers and flow cytometry analysis

HLA-A2/peptide multimers were synthesized as described (1, 14) using peptides MAGE-A10<sub>254-262</sub> (GLYDGM EHL, Ref. 15), Melan-A<sub>26-35</sub> A27L (ELAGIGILTV, Ref. 16) or NY-ESO-1<sub>157-165</sub> C165A (SLLMWITQA, Ref. 17). All Abs were obtained from BD Biosciences (San Jose, CA). For multimer binding assay T cell clones were incubated with the indicated concentration of multimers for the indicated incubation time in PBS, 0.2% BSA, and 0.02% sodium azide (staining and washing buffer). Cells were then washed with the same buffer and immediately analyzed using a FACScan (BD Biosciences) or fixed with PBS, 1% formaldehyde, 2% glucose, and 0.3% sodium azide (fixing buffer) for later analysis. Data analysis was performed using CellQuest software (BD Biosciences).



**FIGURE 2.** Efficiency of staining of MAGE-A10-specific T cell clones with A2/MAGE-A10 peptide fluorescent multimers. Relative efficiency of staining was assessed on MAGE-A10-specific clones and on a control clone specific for an unrelated peptide from influenza matrix (NM55) after incubation with serial dilutions of A2/MAGE-A10 peptide fluorescent multimers 1 h at room temperature (*A*) or under the indicated conditions of time, temperature, and multimer dose (*B*) as detailed in *Materials and Methods*.

*MHC/peptide multimer dissociation assays*

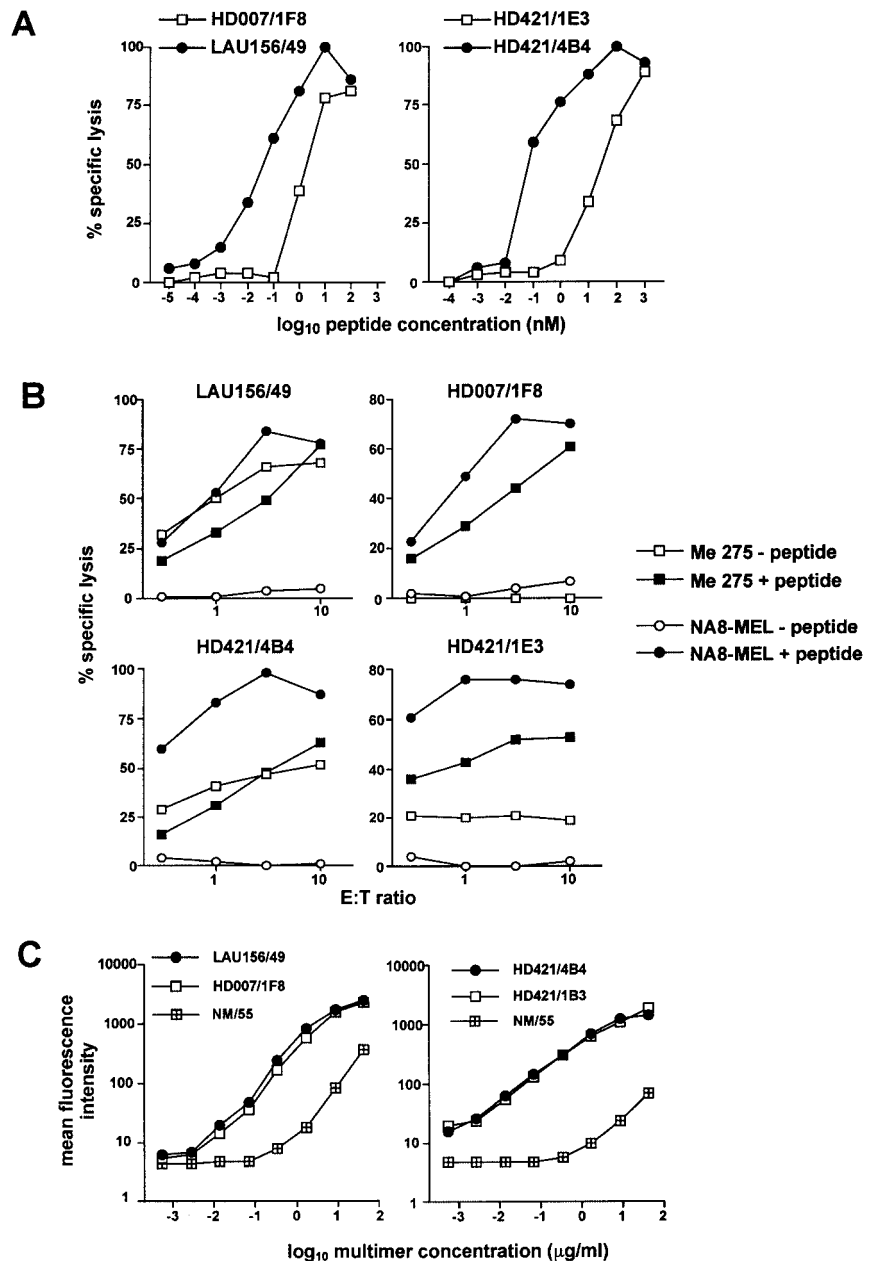
For dissociation experiments T cells were stained with multimers at the indicated dose during 2 h at room temperature. Cells were then washed two times (at 4°C) in 1 ml/sample to eliminate unbound multimers and resuspended in the same buffer. An aliquot (corresponding to  $t_0$ ) was taken and the incubation was then pursued for an additional 90 min at room temperature in the presence of an excess (75  $\mu\text{g/ml}$ ) of unlabeled multimers to avoid rebinding of PE-labeled multimers after their dissociation from the TCR. During this period, aliquots of cells were collected at different time points, washed, and fixed before analysis by flow cytometry. Intensity of multimer fluorescence at each time point was expressed as the percentage of multimer fluorescence at time  $t_0$ .

**Results and Discussion**

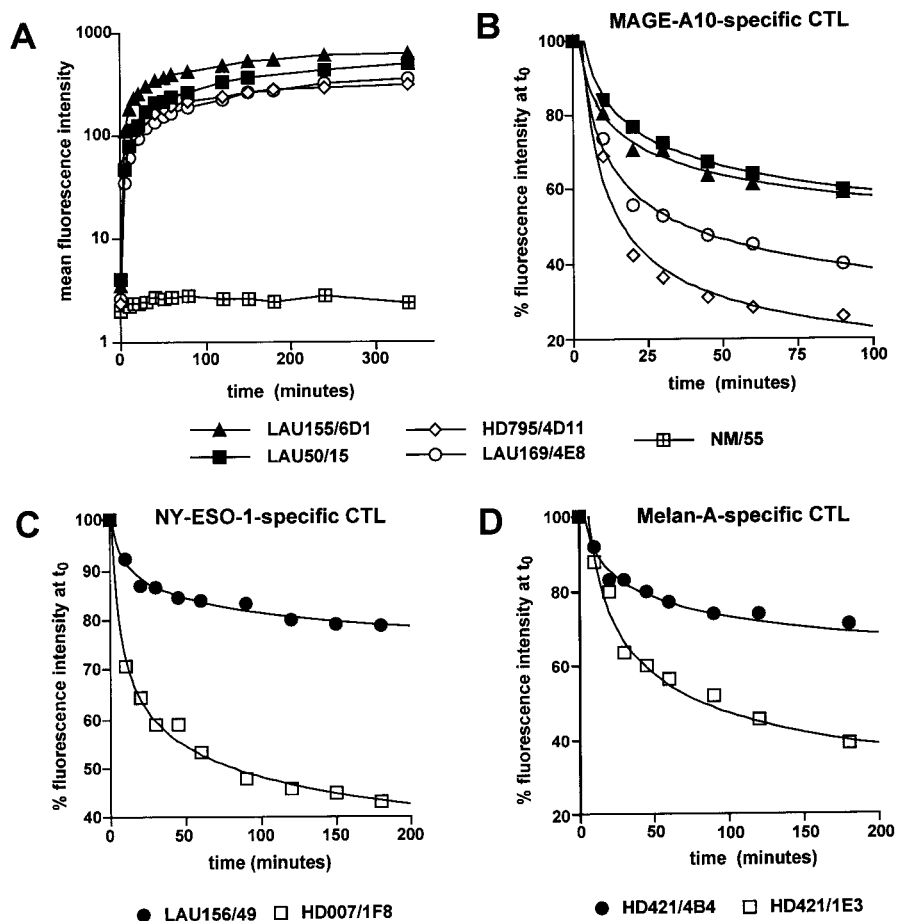
*Efficiency of staining of specific CTL clones with multimers varies with staining conditions and does not always correlate with functional avidity of Ag recognition*

Functional avidity of Ag recognition and efficiency of staining with multimers was initially compared for four CTL clones spe-

cific for peptide MAGE-A10<sub>254-262</sub> (15). The CTL clones were all derived from different individuals and displayed different  $\beta$  variable and/or CDR3 region sequences as previously reported (7). Clones LAU155/6D1 and LAU50/15 (Fig. 1A) recognized peptide MAGE-A10<sub>254-262</sub> with relatively high avidity (50% maximal target cell lysis required was  $\sim 500$  pM) and were able to efficiently kill the MAGE-A10-expressing tumor cell line Me 275 (Fig. 1B). In contrast, CTL clones LAU169/4E8 and HD795/4D11 required significantly higher concentrations of peptide to achieve 50% maximal target cell lysis ( $\sim 100$  nM) and failed to kill Me 275 cells (Fig. 1). Functional avidity also directly correlated with the level of Ag-induced TCR down-regulation (data not shown). Relative efficiency of staining with A2/MAGE-A10<sub>254-262</sub> peptide multimers on specific clones was measured under different conditions of incubation time and temperature (Fig. 2). All clones analyzed expressed comparable levels of TCR and CD8 as assessed by staining with specific mAbs (data not shown). Both the efficiency of



**FIGURE 3.** Functional avidity of Ag recognition, tumor reactivity, and relative multimer staining efficiency of NY-ESO-1- and Melan-A-specific CTL clones. *A*, Melan-A-specific and NY-ESO-1-specific clonal populations were tested for recognition of peptide Melan-A<sub>26-35</sub> A27L or NY-ESO-1<sub>157-165</sub> C156A according to their specificity on T2 cells as described in Fig. 1. *B*, Tumor recognition was similarly assessed at the indicated lymphocyte:target cell ratios by using as target cells tumor cell lines Me 275 (HLA-A2<sup>+</sup>Melan-A<sup>+</sup>NY-ESO-1<sup>+</sup>) and NA8-MEL (A2<sup>+</sup>Melan-A<sup>-</sup>NY-ESO-1<sup>-</sup>) in the absence or in the presence of the corresponding peptide (1  $\mu\text{M}$ ). *C*, Relative efficiency of staining by fluorescent A2/peptide multimers incorporating the corresponding peptide was assessed on NY-ESO-1-, Melan-A-specific clones, and on clone NM55 after 1 h at room temperature as detailed in *Materials and Methods*.



**FIGURE 4.** Avidity of Ag recognition directly correlates with the rate of dissociation of multimers from the TCR in three distinct antigenic systems. *A*, The kinetic of association of multimers on CTLs was first determined for each clone as detailed in *Materials and Methods* by measuring the intensity of staining resulting from the incubation with multimers for the indicated time period. *B*, After staining CTL clones with multimers (at 0.5  $\mu\text{g}/\text{ml}$ ), decay of staining over time in the presence of an excess of unlabeled multimers was determined as detailed in *Materials and Methods*.

staining with A2/MAGE-A10<sub>254–262</sub> peptide multimers on Ag-specific monoclonal population and the background staining measured on a monoclonal population of unrelated specificity were dose dependent (Fig. 2A). As illustrated in Fig. 2B, for clones LAU169/4E8, HD795/4D11, and LAU155/6D1 the efficiency of multimer staining directly correlated with functional avidity and only marginally varied with staining conditions in the case of the first two clones, whereas it increased with temperature and particularly with incubation time in the case of the latter. For clone LAU50/15 staining efficiency at 4°C was comparable to that of low-avidity clones irrespectively of the incubation period (Fig. 2B). However, as in the case of clone LAU155/6D1, the fluorescence signal increased with the incubation temperature and even more significantly with time, approaching, upon incubation during 4 h at 37°C, levels comparable to the ones obtained with the latter. Qualitatively, similar results were obtained using different doses of multimers (Fig. 2). It is also of note that differences in the dose of multimers required to obtain comparable fluorescence intensities for the different populations analyzed were in general relatively modest, irrespectively of the staining condition used (Fig. 2A and data not shown), when compared with the differences in the dose of antigenic peptide required to obtain half maximal lysis (Fig. 1A). The molecular bases of the relative inefficiency of clone LAU50/15 to bind multimers at 4°C as well as of its improved ability to do so at higher temperature and upon prolonged incubation periods are not immediately obvious. Accumulating experimental evidence clearly indicates that molecular factors that can affect multimer staining are more numerous and their effects more complex than anticipated. In particular, integrity of lipid rafts has been recently shown to significantly influence multimer binding to

CD8<sup>+</sup> T cells (10, 11). Thus, an attractive hypothesis to explain the differences between clones LAU155/6D1 and LAU50/15 is that in the case of the first both TCR and/or coreceptor molecules would already be present on the cell surface in association with lipid rafts before interaction with multimers, whereas in the case of the latter such association would mostly take place after interaction with multimers.

We additionally compared efficiency of multimer staining and functional avidity of Ag recognition in two other distinct antigenic systems using NY-ESO-1-specific (17) and Melan-A-specific (16) CTL clones. Clones LAU156/49 (NY-ESO-1 specific) and HD421/4B4 (Melan-A specific) recognized the corresponding antigenic peptides with relatively high avidity and were able to efficiently kill tumor cells expressing the corresponding Ags (Fig. 3, A and B), whereas clones HD007/1F8 (NY-ESO-1 specific) and HD421/1E3 (Melan-A specific) achieved 50% maximal target cell lysis at higher peptide concentrations and failed to efficiently kill Ag-expressing tumor cell lines. However, no significant differences in the staining efficiency of high- and low-avidity CTLs were detectable for both NY-ESO-1- and Melan-A-specific CTL upon incubation during 1 h at room temperature (Fig. 3C).

*Avidity of T cell recognition directly correlates with the rate of dissociation of multimers from the TCR*

Kinetic models of TCR-ligand interaction, supported by several lines of experimental evidence (18–20), have indicated that the potency of a ligand is primarily determined by the duration of its interaction with the TCR. To evaluate the applicability of this concept to the identification of high-avidity CTLs, we measured the relative stability of multimer binding to CTL clones displaying



different functional avidity. We first analyzed the interaction of multimers with CTL clones specific for the MAGE-A10 Ag over time. Incubation at room temperature resulted in a rapid increase of the mean fluorescence for all populations analyzed within the first 2 h and only moderately increased thereafter (Fig. 4A). Similar results were obtained for NY-ESO-1- and Melan-A-specific clones (data not shown). Ligand dissociation from the TCR was measured as decay of multimer staining over time in the presence of an excess of unlabeled multimers as detailed in *Materials and Methods*. It is of note that for the clonal populations used in this study, decay of multimer staining in the absence of cold multimer addition was only minor to undetectable (data not shown). In several other studies, mAb directed against MHC class I molecules have been used to prevent rebinding of dissociated multimers. When using this method we noticed that anti-MHC class I mAb could, depending on the experimental condition and in a clone-dependent fashion, either inhibit or enhance multimer binding (our unpublished observations). Therefore, we routinely used cold multimers to compete over labeled multimers<sup>PE</sup> when measuring kinetics of staining decay.

Remarkably, within each antigenic system, TCR/multimer off-rates directly correlated with functional avidity being faster for relatively low-avidity as compared with relatively high-avidity CTL clones (Fig. 4). Interestingly, this was not only the case when analyzing monoclonal populations, as we obtained similar results in the case of polyclonal monospecific populations derived from a melanoma patient who developed a strong Ag-specific response accompanied by functional avidity maturation and improved tumor recognition upon immunization with a CTL-defined synthetic vaccine.<sup>4</sup> Similar observations have recently been reported by others (21, 22). Binding of MHC/peptide complexes to TCR is only the first of a series of complex molecular events leading to T cell activation, which can, to some extent, differ for different T cell clones. Thus, for some clones, functional avidity of Ag recognition could dissociate from TCR/multimer off-rates. For example, a CTL clone specific for peptide tyrosinase<sub>368–376</sub> formed TCR/multimer complexes of relatively low stability as compared with other clones displaying an apparent comparable functional avidity (8). In contrast, increased stability of TCR/multimer complex interaction can lead to decreased Ag recognition (9). However, the data reported in this work and by others (18–20, 23, 24) suggest that such discrepancies would represent exceptions rather than the general rule.

### Conclusions

Whereas the availability of fluorescent MHC/peptide multimers has tremendously improved our ability of detecting and isolating tumor Ag-specific T cells, the results reported above and in other studies (5, 8, 9) indicate that either no significant differences in the efficiency of multimer staining of tumor Ag-specific clonal populations displaying different functional avidity of Ag recognition and tumor reactivity, or differences that do not directly correlate with the latter, are common findings. However, our data clearly indicate that a better evaluation of the relative ability of tumor Ag-specific CTL to recognize and destroy tumor cells is based on the measure of TCR dissociation kinetics from multimers incorporating synthetic peptides corresponding to naturally processed tumor Ag peptides. Analyzing dissociation rates of multimers from TCRs of CD8<sup>+</sup> T cell populations under test could considerably improve the qualitative value of multimer-based molecular monitoring of clinical trials of cancer vaccination with CTL-defined immunogens and could be instrumental for the isolation of tumor

Ag-specific CTL populations with high functional avidity and tumor reactivity to be used for adoptive transfer therapy.

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