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*J Immunol* (2003) 170 (10): 5110–5117.

<https://doi.org/10.4049/jimmunol.170.10.5110>

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# Decreased Binding of Peptides-MHC Class I (pMHC) Multimeric Complexes to CD8 Affects Their Binding Avidity for the TCR But Does Not Significantly Impact on pMHC/TCR Dissociation Rate<sup>1</sup>

Valérie Dutoit,<sup>2\*†</sup> Philippe Guillaume,<sup>2‡</sup> Maha Ayyoub,<sup>\*†</sup> Charles S. Hesdorffer,<sup>†</sup> Immanuel F. Luescher,<sup>‡</sup> and Danila Valmori<sup>3\*†</sup>

The CD8 coreceptor plays a crucial role in both T cell development in the thymus and in the activation of mature T cells in response to Ag-specific stimulation. In this study we used soluble peptides-MHC class I (pMHC) multimeric complexes bearing mutations in the CD8 binding site that impair their binding to the MHC, together with altered peptide ligands, to assess the impact of CD8 on pMHC binding to the TCR. Our data support a model in which CD8 promotes the binding of TCR to pMHC. However, once the pMHC/TCR complex is formed, the TCR dominates the pMHC/TCR dissociation rates. As a consequence of these molecular interactions, under physiologic conditions CD8 plays a key role in complex formation, resulting in the enhancement of CD8 T cell functions whose specificity, however, is determined by the TCR. *The Journal of Immunology*, 2003, 170: 5110–5117.

**C**D8<sup>+</sup> CTL are the main effector arm of the cellular immunity against intracellular pathogens and neoplasms and are also involved in the pathogenesis of some autoimmune disorders. At the molecular level, recognition of Ag-derived peptides-MHC class-I (pMHC)<sup>4</sup> complexes on the surface of APC or target cells takes place through receptor-ligand (pMHC/TCR) and auxiliary molecule interactions (including that of the coreceptor CD8, costimulatory, and adhesion molecules) that together contribute to the formation of the immunological synapse (1–3).

In the case of CTL, the presence of CD8 augments the pMHC/TCR interaction in living cells. The molecular and mechanistic bases of CD8-mediated enhancement of T cell function have been extensively investigated. It has been shown that T cell activation is facilitated by coordinated binding of CD8 and TCR by the same pMHC molecule. Albeit, most data support this notion, resulting in the classification of CD8 as a coreceptor; it has been suggested that Ag-independent binding of CD8 to MHC molecules may occur (4). Two distinct, but not mutually exclusive, mechanisms have been put forward to explain CD8 coreceptor function (5, 6). The first is that binding of CD8 to conserved residues of the MHC class I H chain stabilizes the interaction between the TCR and pMHC com-

plex and enhances T cell activation by increasing the  $t_{1/2}$  of the TCR/CD8/pMHC complex (7). The second is that association of CD8 $\alpha$ , through its cytoplasmic tail, with p56<sup>lck</sup> contributes to the recruitment of this tyrosine kinase to the TCR/CD3 complex where it is critical for early pMHC-mediated phosphorylation reactions (8).

In this study we assessed the impact of CD8 on pMHC/TCR kinetics by using fluorescent multimeric complexes (multimers) of HLA-A\*0201 (A2) molecules bearing mutations in the  $\alpha 3$  domain known to diminish or abrogate the CD8/pMHC interaction, containing either parental or altered peptide ligands. Multimers containing an A2 A245V single mutant, known to bind CD8 with decreased affinity compared with the wild-type molecule, showed a strongly decreased capacity to bind high-avidity tumor-Ag-specific CD8<sup>+</sup> T cells in different antigenic systems (9). Multimers containing the D227K or D227K/T228A mutations that abrogate A2 interaction with CD8 (10, 11) showed low to undetectable binding, depending on the CD8<sup>+</sup> T cell clone analyzed. Interestingly, the association kinetics of A245V multimers containing the parental peptide was similar to that of wild-type multimers containing a weak agonist peptide. However, measurement of TCR/multimer dissociation kinetics revealed that, in contrast to the accelerated dissociation rate displayed by multimers containing the weak agonist as compared with those containing the parental peptide, complexes formed by the TCR with either wild-type or mutated multimers incorporating the parental peptide displayed similar stability. Together, our data suggest a model in which CD8 promotes the association of pMHC with the TCR, whereas the TCR dominates the pMHC/TCR complex dissociation rate.

## Materials and Methods

### Cells and Ag recognition assay

Monoclonal tumor-Ag-specific CD8<sup>+</sup> T cell populations were obtained from circulating lymphocytes or tumor infiltrating lymphocytes of cancer patients, as previously described (12). The influenza matrix-specific clone NM55 was obtained from circulating lymphocytes of an A2-expressing healthy donor. Clonal populations were expanded by periodic stimulation with PHA (Sigma-Aldrich, St. Louis, MO) and irradiated allogeneic feeder cells. Ag recognition was assessed using chromium release assay (CTL assay). The A2<sup>+</sup> human mutant cell line CEMx721.T2 (T2) (13) or the

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Received for publication December 13, 2002. Accepted for publication March 4, 2003.

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<sup>1</sup> D.V. and M.A. were partially supported by the Cancer Research Institute.

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<sup>4</sup> Abbreviations used in this paper: pMHC, peptides-MHC class I; A2, HLA-A\*0201; T2, A2<sup>+</sup> human mutant cell line CEMx721.T2;  $\beta_2$ -m,  $\beta_2$ -microglobulin.

melanoma cell lines Me 275 (A2<sup>+</sup> Melan-A<sup>+</sup> MAGE-A10<sup>+</sup>) and NA8-MEL (A2<sup>+</sup> Melan-A<sup>-</sup> MAGE-A10<sup>-</sup>) were used as targets. Briefly, after labeling with <sup>51</sup>Cr for 1 h at 37°C, followed by extensive washing, target cells (1000/well) were incubated with effector cells at the indicated E:T ratio for 4 h at 37°C in V-bottom microwells. In peptide titration experiments, target cells were incubated with effectors at an E:T ratio of 10:1 in the presence of serial dilutions of the indicated peptide. Chromium release was measured in the supernatant of the cultures using a gamma counter. The percentage of specific lysis was calculated as  $100 \times [(\text{experimental} - \text{spontaneous release}) / (\text{total} - \text{spontaneous release})]$ . Inhibition of Ag recognition by anti-CD8 Abs was assessed by incubating CD8<sup>+</sup> T cell clones with the corresponding peptides at a concentration giving 50% of maximal lysis together with serial dilutions of anti-CD8 mAb (RPA-T8; BD Biosciences, San Diego, CA).

### pMHC multimers

Wild-type and A245V, D227K, or D227K/T228A mutated A2/peptide complexes incorporating peptides Melan-A<sub>26–35A27L</sub> analog (ELAGIGILTV) (14), MAGE-A10<sub>254–262</sub> parental (GLYDGMEHL) (15), or single-A substituted analogs were prepared as described (16). The A2 H chain mutants were produced by PCR mutagenesis using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA). Recombinant soluble wild-type or mutant human HLA H chains and  $\beta_2$  microglobulin ( $\beta_2$ -m) were obtained using a prokaryotic expression system (pET; R&D Systems, Minneapolis, MN). The H chain was modified by COOH-terminal addition of a peptide sequence containing the BirA enzymatic biotinylation site. The HLA H chain,  $\beta_2$ -m, and peptide were refolded by dilution. The 45-kDa refolded product was purified by fast liquid chromatography and then biotinylated by recombinant BirA (Avidity, Denver, CO) in the presence of biotin, ATP, and Mg<sup>2+</sup> (all from Sigma-Aldrich). The degree of biotinylation was estimated by gel migration in the presence of avidin. The concentration of the pMHC monomer was calculated by Bradford measurement of optical density at 595 nm in the presence of phosphoric acid, using BSA as standard. Monomers were aliquoted (50  $\mu$ g) and stored at  $-80^\circ\text{C}$ . Multimers were prepared by addition of PE-streptavidin at a 1:4 molar ratio and stored at 4°C. Correct folding of pMHC complexes was assessed by ELISA using the mAb w6/32, as described (17). Briefly, multimers (10, 1, or 0; 1  $\mu$ g/ml) were attached to 96-well microtiter plates (Maxisorb; Nunc, Wiesbaden, Germany) previously coated with the anti- $\beta_2$ -m mAb B1G3 (Beckman Coulter, Krefeld, Germany). Biotin-conjugated mAb w6/32, recognizing a monomorphic determinant on MHC class I molecules that is dependent on the correct folding of the pMHC H chain/ $\beta_2$ -m complex, was used as secondary Ab. The assay was then conducted by adding avidin-peroxidase and developed with tetramethylbenzidine. The reaction was terminated by adding H<sub>2</sub>SO<sub>4</sub>. The OD was measured at 450 nm. All multimers used in this study were stable under the experimental conditions used; after storage at 4°C for several months, staining of specific clones was comparable to that obtained just after synthesis. In addition, preincubation at room temperature for 2 h did not significantly modify the staining of specific T cells.

### Assessment of pMHC multimer binding to and dissociation from Ag-specific CD8<sup>+</sup> T cell clones

For multimer binding assay, CD8<sup>+</sup> T cell clones were incubated with multimers at the indicated dose for the indicated time period in PBS containing 0.2% BSA, 0.02% sodium azide (staining and washing buffer). For dissociation assay, CD8<sup>+</sup> T cell clones were incubated with multimers at a dose giving a mean fluorescence intensity of  $\sim 200$  for 2 h at room temperature and washed at 4°C to eliminate unbound multimers. An aliquot (corresponding to time zero ( $t_0$ )) was taken at that time, and the incubation was then pursued at room temperature in the presence of an excess of unlabeled multimers incorporating the parental peptide. During this period, aliquots were collected at different time points. Inhibition of multimer binding by anti-CD8 mAb (RPA-T8; BD Biosciences) was assessed by coincubating CD8<sup>+</sup> T cell clones with multimers at a concentration giving a mean fluorescence intensity of  $\sim 200$  together with serial dilutions of anti-CD8 mAb. Samples were analyzed using a FACScan (BD Biosciences). Data analysis was performed using CellQuest software (BD Biosciences).

### Assessment of T cell activation following interaction between TCR and wild-type or mutated multimers

TCR down-regulation was assessed upon incubation of T cells with multimers<sup>PE</sup> at the indicated dose and for the indicated time period, followed by staining with anti-CD3<sup>FITC</sup> mAb (BD Biosciences) for 20 min at 4°C. Simultaneous incubation with multimers and anti-CD3 at  $t_0$  did not result in any significant inhibition of staining compared with incubation with

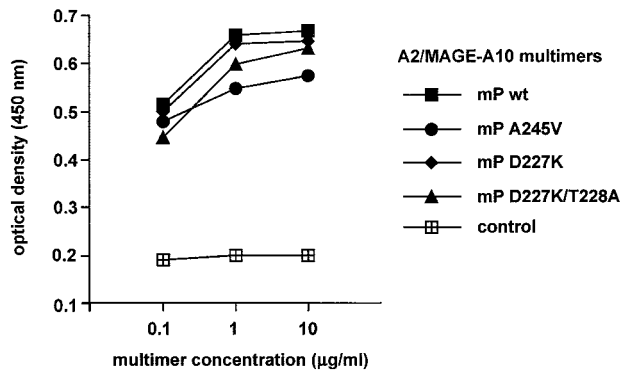
anti-CD3 alone. Bystander lysis of P815 mastocytoma cells was assessed by CTL assay as described above by incubating <sup>51</sup>Cr-labeled P815 cells (1000/well) with CD8<sup>+</sup> T cell clones at the lymphocyte to target cell ratio of 10:1 for 4 h at 37°C in the presence of serial dilutions of the indicated multimers. Incubation of CTL with a dose of antigenic peptide corresponding to that incorporated in the multimers did not result in detectable bystander killing. Cytokine release was determined by ELISPOT (18) using an IFN-ELISPOT kit (Mabtech, Stockholm, Sweden) according to the manufacturer's instructions, with minor modifications. Briefly, T cells (5000/well) were incubated in nitrocellulose-lined 96-well microplates (Millipore MAHA S45; Millipore, Bedford, MA) previously coated overnight with Ab to human IFN- $\gamma$  together with different concentrations of either wild-type or mutated multimers or the appropriate synthetic peptide as internal control. After incubation for 20 h at 37°C, cells were removed and plates were developed with a second (biotinylated) Ab to human IFN- $\gamma$  and streptavidin-alkaline phosphatase and then developed with tetramethylbenzidine. Spots were counted using a stereomicroscope with a magnification of  $\times 15$ . Incubation of CTL with a dose of antigenic peptide corresponding to that incorporated in the multimers did not result in detectable IFN- $\gamma$  secretion.

## Results and Discussion

### Mutations in the A2 $\alpha 3$ CD8-binding domain impact on the binding of pMHC multimeric complexes to tumor-Ag-specific T cell clones and on stimulation of their effector functions

The binding between human CD8 $\alpha$  homodimers and the MHC class I molecule has been elucidated by an analysis of the crystal structure of the CD8 $\alpha$ /A2 complex (19). Whereas these studies have shown that CD8 binding involves contacts with the  $\alpha 2$ ,  $\alpha 3$ , and  $\beta_2$ -m domains of the pMHC molecule, adhesion measurements between CD8 and A2 point mutants have identified clusters of residues in the  $\alpha 3$  domain playing a dominant role in the interaction (11). Based on this and additional information (9, 10), we introduced in the  $\alpha 3$  domain of the A2 molecule mutations known to affect binding to CD8 to different extents. One of these was A245V. This mutation corresponds to a natural polymorphism in the  $\alpha 3$  domain. The product of the corresponding allele (*HLA-A68*) is a poor ligand for CD8 (20). The A245V A2 mutant was previously shown to bind CD8 with over 10-fold decreased affinity compared with the wild-type molecule (9). This decreased binding appeared to be the consequence of a small but significant distortion of the 223–229 loop in the  $\alpha 3$  domain that is directly involved in contacting CD8 (9). Other mutations (D227K, or D227K/T228A) were then directly introduced in the 223–229 loop. The D227K/T228A double mutation was previously shown to abrogate A2 binding to CD8 (10).

Wild-type and mutated A2/peptide multimers were synthesized, incorporating A2-restricted peptides derived from tumor-associated Ags, namely MAGE-A10<sub>254–262</sub> (15) and Melan-A<sub>26–35A27L</sub> analog (14). Correct folding of pMHC complexes was assessed by ELISA using the mAb w6/32, as described (17) (Fig. 1). MAGE-A10- and Melan-A-specific CD8<sup>+</sup> T cell clones displaying high functional avidity of Ag recognition and tumor reactivity (Fig. 2, A and B) were used to assess the impact of mutations in the CD8 binding site on multimer binding to specific T cells. Recognition of Ag by these clones was completely inhibited by anti-CD8 mAbs (Fig. 2C). Specific multimer binding to CD8<sup>+</sup> T cell clones was assessed by incubating T cells for 1 h at room temperature in the presence of serial multimer dilutions. The CD8<sup>+</sup> T cell clone NM55, specific for an irrelevant A2-binding peptide (Flu-MA<sub>58–66</sub> from the influenza matrix protein), was used for internal control. As illustrated in Fig. 3A, multimers containing the A2 A245V single mutant showed a highly decreased binding avidity on the MAGE-A10-specific clone 6D1 (21). Reduction in the specific binding avidity of A245V vs wild-type multimers was also observed in the case of Melan-A-specific clone 17 as well as in the

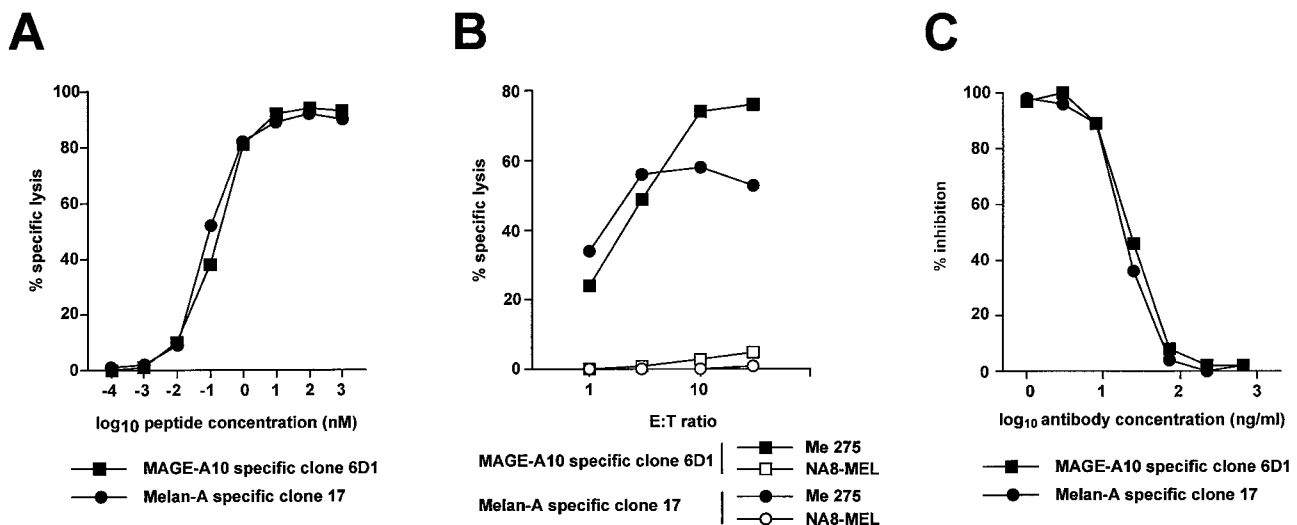


**FIGURE 1.** A2/peptide multimers bearing mutations in the CD8 binding domain are correctly folded. Wild-type (wt) and mutated A2/peptide multimers incorporating peptide MAGE-A10<sub>254–262</sub> were attached to 96-well microtiter plates using an anti- $\beta_2$ -m mAb as described in *Materials and Methods*. Correct folding was evaluated using the mAb w6/32 in an ELISA. For internal control, the plate was coated with the anti- $\beta_2$ -m mAb alone. Similar results were obtained with multimers incorporating peptide Melan-A<sub>26–35A27L</sub> and when using the A2-specific mAb HB82. mP, multimer incorporating the parental MAGE-A10 peptide.

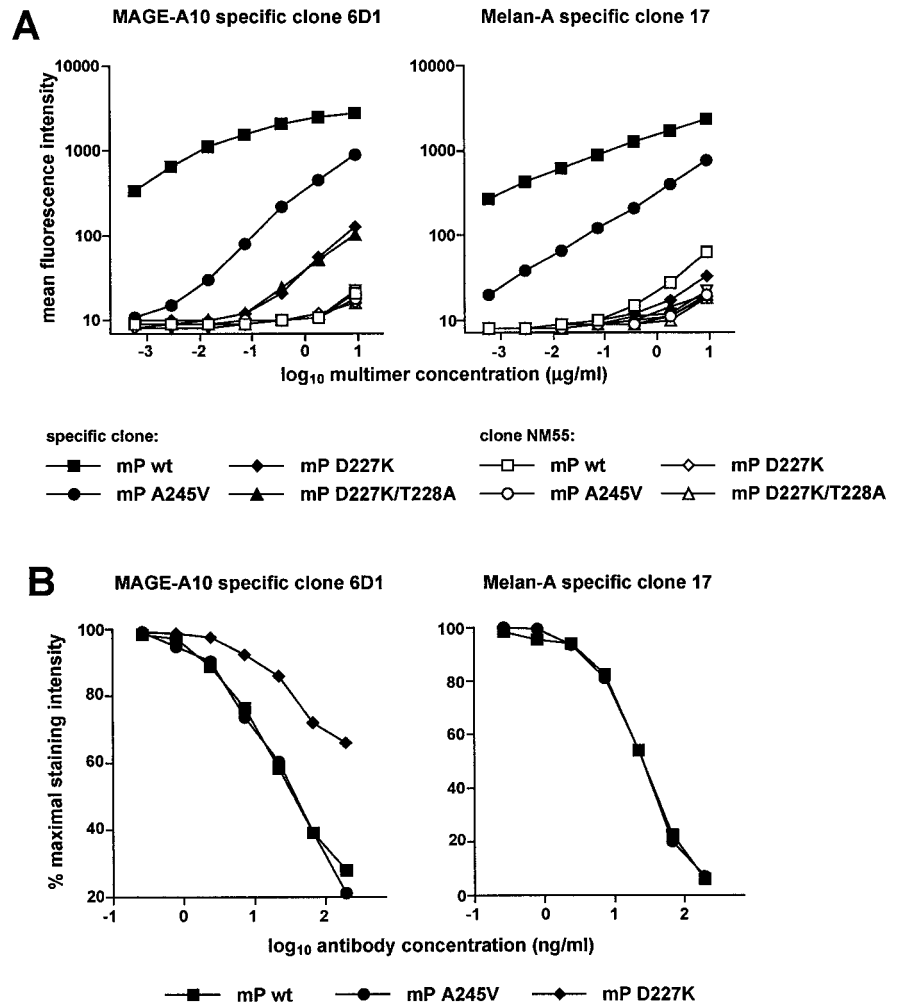
case of other MAGE-A10- and Melan-A-specific clones that recognize the peptide with lower avidity (data not shown). Similar results were obtained upon staining at 4°C, albeit the mean fluorescence intensity of multimer staining was, in this case, generally lower (data not shown). Multimers containing the D227K or D227K/T228A mutations showed low specific binding on clone 6D1 and no specific binding to clone 17. No specific binding was detected with multimers containing the D227K or D227K/T228A mutations on other MAGE-A10-specific clones that recognize the peptide with lower avidity (data not shown). It is noteworthy that binding of A245V multimers to tumor-Ag-specific CD8<sup>+</sup> T cell clones, albeit significantly reduced, was entirely CD8 dependent, as it could be completely inhibited by anti-CD8 mAbs (shown in Fig. 3B). In contrast, D227K multimer binding was only partially inhibited by anti-CD8 mAbs. The effect of the A245V mutation on multimer binding to specific T cells in relation to the relative ef-

iciency of pMHC ligand recognition by TCR was addressed by analyzing the binding capacity of wild-type vs V245A multimers, incorporating single amino acid substituted analogs of peptide MAGE-A10<sub>254–262</sub> (22). As illustrated in Fig. 4A, and similar to A245V multimers incorporating the parental peptide, A245V multimers incorporating full agonist peptides (A1, A2, A4, A8) displayed reduced binding capacity compared with the corresponding wild-type multimers. However, whereas wild-type multimers containing weak agonists (A3, A5, A6, A7, A9) displayed decreased but detectable specific binding, the corresponding A245V multimers failed to significantly bind this CTL clone (Fig. 4A). From these results it could be inferred that reduction of pMHC binding to CD8 in the context of low avidity pMHC/TCR interactions results in undetectable multimer binding. Thus, in principle the use of A245V multimers could be instrumental for the selective isolation of CTL that recognize the Ag with high avidity. However, because of the previously documented lack of direct correlation between efficiency of Ag recognition and avidity of multimer staining (21–23), some clones that recognize the Ag with relatively low avidity and show no reactivity to Ag-expressing tumors (i.e., Melan-A-specific clone 5F1, Fig. 4B) can bind wild-type multimers with relatively high avidity and, therefore, significantly bind A245V-mutated multimers and could be isolated when using the latter. Together, these results show that, as suggested by previous studies (24, 25), interaction with CD8 is crucial for binding of A2/peptide multimers to T cells. In addition, they underline that the impact of CD8 involvement in the detection of significant multimer binding to specific T cells can be more or less critical, depending on the CD8<sup>+</sup> T cell population under analysis and on the avidity of binding of wild-type multimers.

The impact of mutations in the  $\alpha 3$  domain on the ability of A2/peptide multimers to trigger T cell functions was assessed by measuring both early and late T cell activation events following incubation of either wild-type or mutated multimers with T cells. These included the following: 1) TCR down-regulation, 2) stimulation of CTL effector functions, and 3) cytokine production. TCR down-regulation is one of the earliest activation events induced by TCR triggering. As illustrated in Fig. 5A, incubation with serial



**FIGURE 2.** Ag recognition, tumor reactivity, and inhibition of activity by anti-CD8 Abs of MAGE-A10- and Melan-A-specific T cell clones 6D1 and 17. *A*, Clonal populations were tested for peptide recognition in chromium release assay using T2 cells as targets at a lymphocyte to target ratio of 10:1 in the presence of serial dilutions of the corresponding peptide. *B*, Tumor recognition was similarly assessed at the indicated lymphocyte to target cell ratios by using as target cells tumor cell lines Me 275 (A2<sup>+</sup>, Melan-A<sup>+</sup>, MAGE-A10<sup>+</sup>) and NA8-MEL (A2<sup>+</sup>, Melan-A<sup>-</sup>, MAGE-A10<sup>-</sup>) in the absence or in the presence of peptide MAGE-A10<sub>254–262</sub> or Melan-A<sub>26–35A27L</sub> (1  $\mu$ M). *C*, Inhibition of Ag recognition by anti-CD8 Abs was assessed by incubating CD8<sup>+</sup> T cell clones with the corresponding peptides at a concentration giving 50% of maximal lysis together with serial dilutions of anti-CD8 mAb.



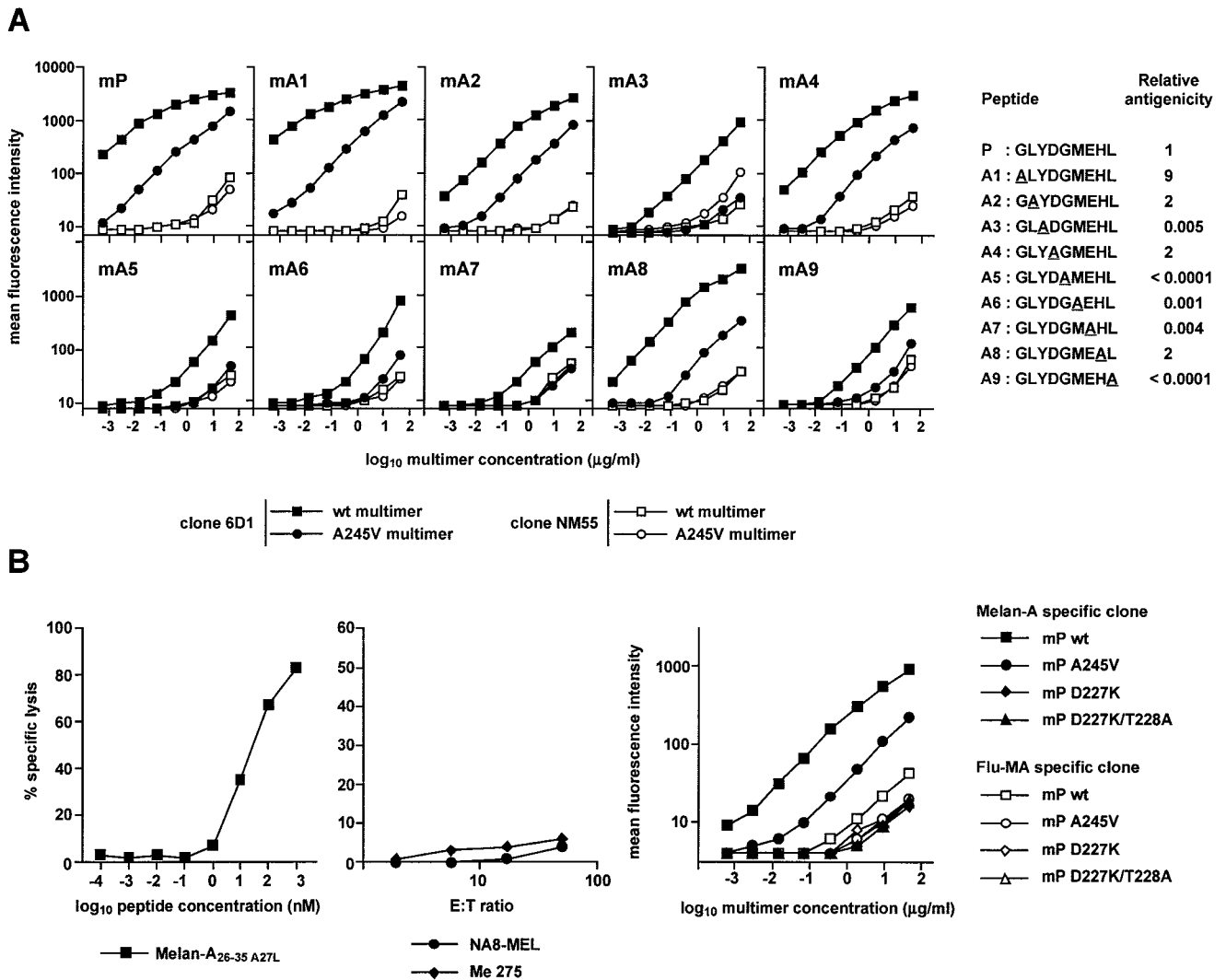
**FIGURE 3.** CD8-dependent interaction of A2/peptide multimers with specific CD8<sup>+</sup> T cell clones. *A*, Binding of wild-type and mutated A2/peptide multimers to specific CD8<sup>+</sup> T cells is shown for MAGE-A10-specific clone 6D1 and Melan-A-specific clone 17 after incubation for 1 h at room temperature with serial dilutions of multimers. Clone NM55, specific for the influenza matrix protein derived peptide Flu-MA, was used as an internal control. *B*, Inhibition of A2/peptide multimer binding by anti-CD8 Abs. Clones were incubated with wild-type or mutated A2/MAGE-A10 multimers at a concentration giving a mean fluorescence of ~200 in the presence of serial dilutions of the anti-CD8 mAb. Results are shown as a percentage of maximal staining intensity. Preincubation with anti-CD8 mAb for 30 min followed by removal of unbound mAb by washing and incubation with multimers gave similar results.

dilutions of wild-type multimers for 2 h at 37°C induced a high level of TCR down-regulation in a dose-dependent fashion, both in the case of MAGE-A10- and Melan-A-specific clones. A245V mutated multimers also induced TCR down-regulation, although to a lesser extent. In contrast, no detectable TCR down-regulation was induced by either D227K or D227K/T228A multimers. The effect of mutations in the  $\alpha 3$  domain on multimer mediated triggering of T cell effector functions was assessed in a functional assay upon incubation of CTL with either wild-type or mutated multimers in the presence of <sup>51</sup>Cr-labeled A2<sup>-</sup> P815 mastocytoma cells. As shown in Fig. 5*B*, wild-type multimers efficiently activated both clones 6D1 and 17, resulting in bystander killing of P815 cells. Decreased but significant effector function was triggered by A245V multimers, whereas both D227K and D227K/T228A multimers failed to significantly trigger CTL. Finally, the impact of mutations in the  $\alpha 3$  domain on cytokine production was assessed by IFN- $\gamma$  ELISPOT assay. Again, incubation of both T cell clones with serial dilutions of wild-type multimers and, to a lesser extent A245V multimers resulted in efficient IFN- $\gamma$  secretion (Fig. 5*C*). In contrast, no detectable IFN- $\gamma$  secretion was observed upon incubation with D227K and D227K/T228A multimers. Together, these results are consistent with the important role of CD8 engagement for the stimulation of early to late T cell activation events. They show that the A245V mutation results in a decreased capacity of the multimers to activate early to late activation events. It is noteworthy that in the three different functional assays, an ~10-fold higher dose of A245V compared with wild-type multimers was required to achieve 50% maximal function. In the case of the

more drastic D227K or D227K/T228A mutations, multimer-mediated T cell activation was impaired even when the highest multimer doses were used, such as in the case of clone 6D1 that gave a low but detectable specific binding (Fig. 3).

#### *Decreased pMHC multimer binding to CD8 does not affect pMHC/TCR dissociation rates*

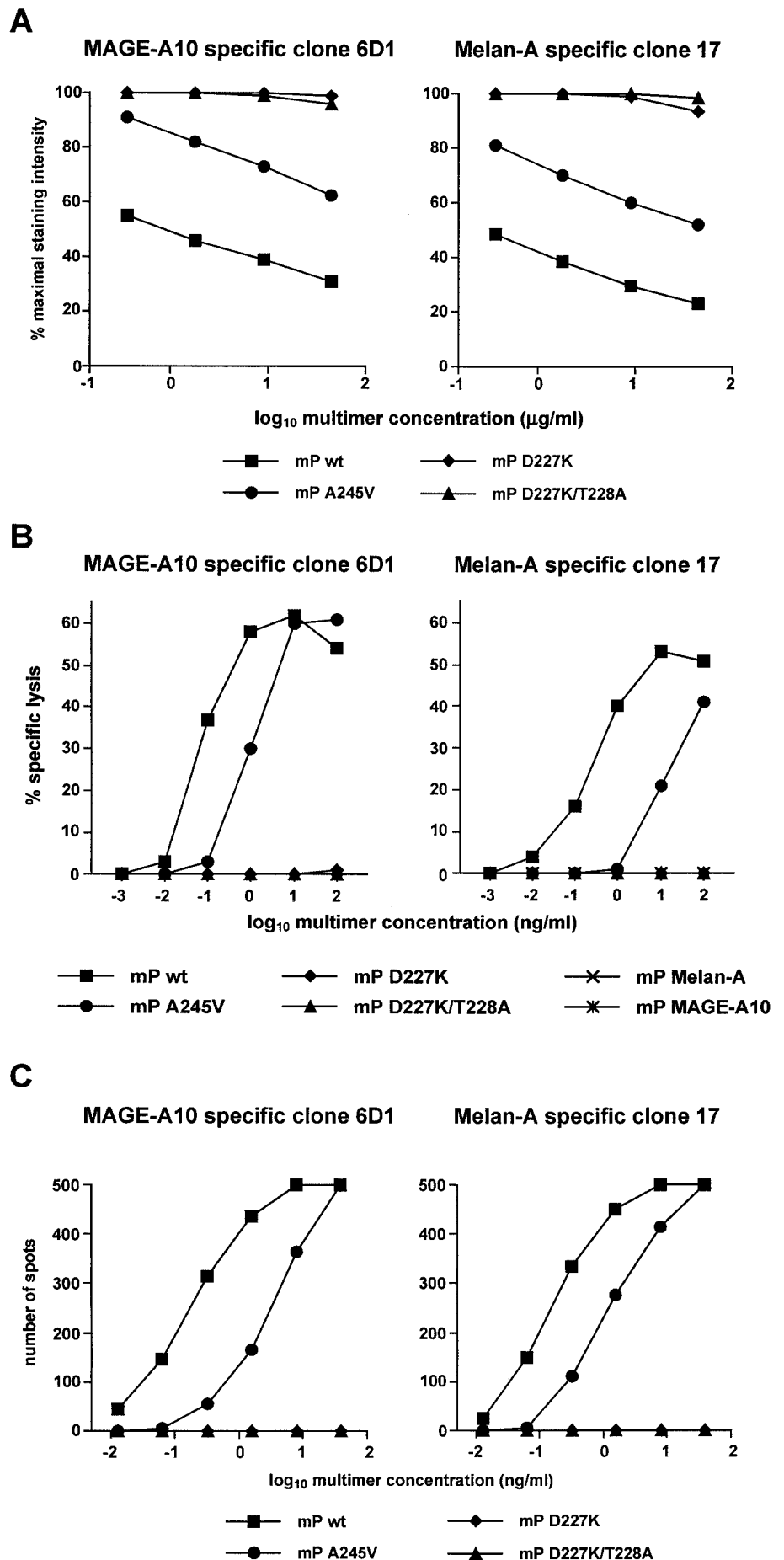
It has been proposed that the potency of a given ligand recognized by the TCR is primarily determined by the off-rate of the TCR-ligand interaction (26–28). According to this model, a minimal time of TCR-ligand interaction (corresponding to a certain off-rate) is required for a complete TCR signal to be sent to the T cell. The ligand in this case is a full agonist, whereas ligands displaying faster off-rates are weak agonists or antagonists (27). The low binding affinity of the TCR for monomeric pMHC ligands (29–31), however, together with their rapid dissociation kinetics ( $t_{1/2}$  between 2 and 30 s at 25°C) (32, 33) has hampered comparison of the interaction kinetics of different pMHC ligands with clonally distributed TCR on living cells under physiologic conditions. The use of multivalent pMHC complexes has allowed us recently to overcome these limitations by increasing the avidity of the pMHC/TCR interaction. In recent studies using pMHC multimers and tumor-Ag-specific clones displaying diverse efficiency of Ag recognition and tumor reactivity, we found a clear correlation between efficiency of recognition and stability of pMHC complex interaction with TCR. Clones recognizing the Ag with lower efficiency displayed, under similar experimental conditions, faster off-rates than clones recognizing the Ag with higher efficiency



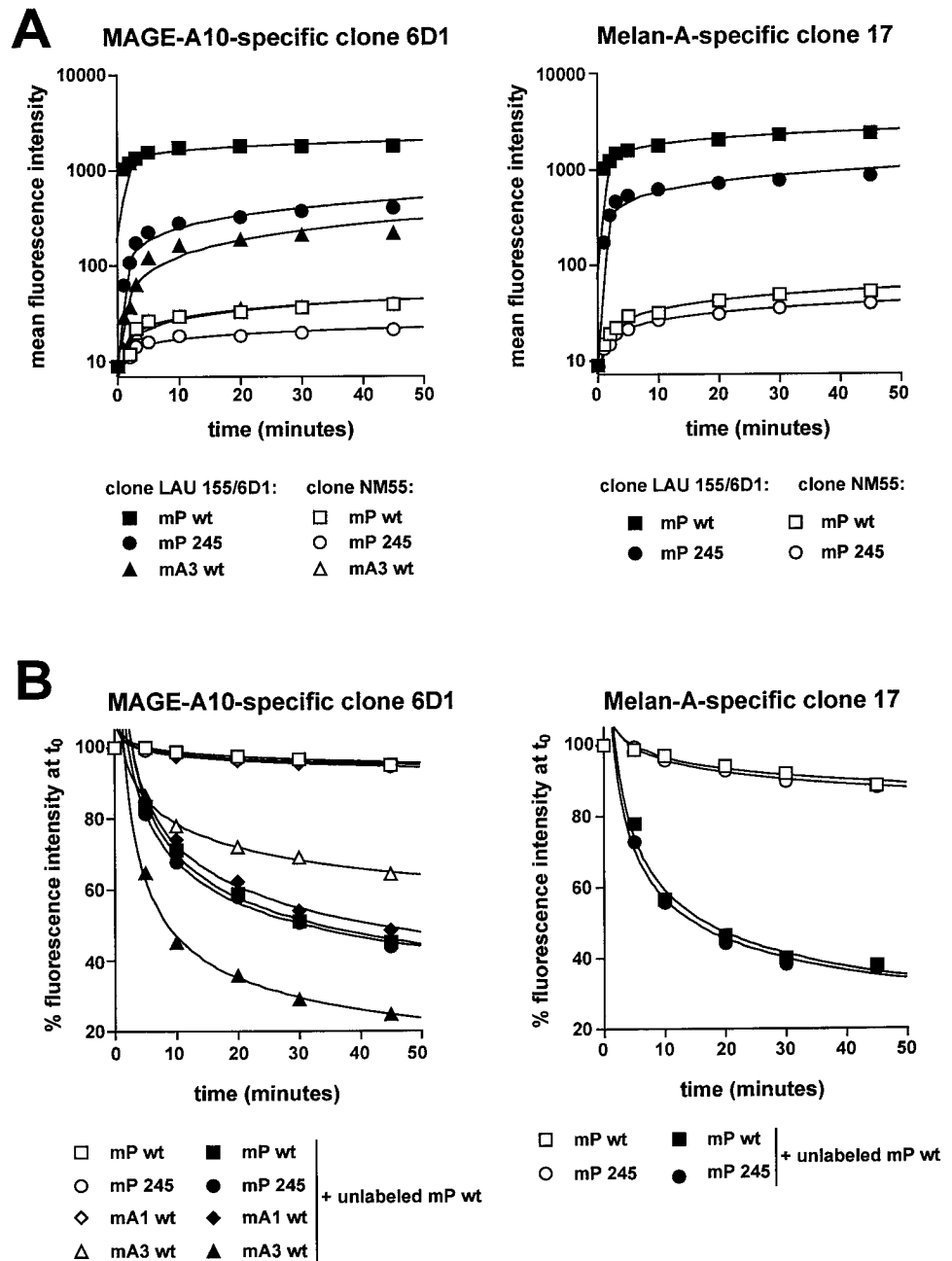
**FIGURE 4.** Impact of efficiency of Ag recognition on the binding of A245V mutated multimers to specific CTL. *A*, The relative binding avidity of wild-type vs A245V A2/MAGE-A10 multimers incorporating the indicated peptide for clone 6D1 was assessed as in Fig. 3A. The activity of peptide variants relative to that of the parental peptide was previously assessed (22) and is shown. *B*, Ag recognition and tumor reactivity of the Melan-A-specific clone 5F1 were assessed as in Fig. 2, *A* and *B*. Binding of wild-type and mutated A2/peptide multimers was assessed as in Fig. 3A.

(21). We also observed that for a given clone, multimers incorporating weak agonist ligands displayed decreased binding efficiency compared with multimers incorporating full agonist ligands and faster off-rates (22). Both pMHC/CD8 binding kinetics and the influence of CD8 on pMHC/TCR interactions have been previously studied. These studies have shown that CD8 binds pMHC with lower affinity and faster kinetics independently from the TCR. Using soluble human recombinant molecules and surface plasmon resonance biosensor (Biacore, Uppsala, Sweden), no direct binding of TCR with CD8 or influence of CD8 on the binding of TCR to pMHC could be demonstrated (34). Because a previous study using murine molecules had shown a slower pMHC/TCR dissociation kinetic in the presence of soluble CD8, it was inferred that CD8 enhances the affinity of TCR for its specific ligand by reducing the pMHC/TCR off-rate (35). Thus, the current view of CD8 coreceptor function, as far as the influence of CD8 binding to pMHC is involved, is still that it stabilizes the interaction between TCR and pMHC complex and enhances T cell activation by increasing the half-life of the TCR/CD8/pMHC complex (5). However, the impact of CD8 binding on pMHC/TCR interaction kinetics on living cells has remained unsolved. To address this issue we compared the kinetics of TCR interaction with wild-type or

A245V pMHC multimers containing the MAGE-A10 parental peptide to those of wild-type multimers containing the weak agonist ligand (A3). Incubation of clone 6D1 with either wild-type or mutated multimers resulted in a rapid increase of the specific mean fluorescence within the first hour, but only a moderate increase thereafter (Fig. 6A). Similar results were obtained for clone 17. Specific ligand dissociation from TCR was measured as decay of multimer staining over time, either in the absence or in the presence of an excess of unlabeled multimers as reported previously (22). Consistent with our previous results (22), for clone 6D1, wild-type multimers incorporating the weak agonist analog (A3) displayed faster pMHC/TCR dissociation rates compared with wild-type multimers containing either the parental MAGE-A10 peptide or a full agonist analog (A1), both in the absence or in the presence of unlabeled multimers (Fig. 6B). In contrast, wild-type and A245V multimers incorporating the parental peptide displayed identical dissociation kinetics. Similarly, in the case of Melan-A-specific clone 17, wild-type and A245V multimers displayed identical dissociation kinetics. Although it cannot be formally excluded that small differences in the dissociation rates of A245V vs wild-type multimers from the TCR are hidden by the multivalency of the pMHC ligand used in this study, our results indicate that such



**FIGURE 5.** Triggering of T cell function by wild-type or mutated multimers. *A*, TCR down-regulation was assessed after incubation of T cells with multimers for 2 h at 37°C followed by staining with anti-CD3. Results are shown as a percent of maximal staining intensity, obtained in the absence of multimers. *B*, Bystander lysis of P815 mastocytoma cells by CD8<sup>+</sup> T cell clones was assessed in a CTL assay at the E:T ratio of 10:1 for 4 h at 37°C in the presence of serial dilutions of the indicated multimers. *C*, Cytokine release was determined by ELISPOT as described in *Materials and Methods*. Results are shown as the number of spots obtained by adding 5000 CTL/well.



**FIGURE 6.** Impact of mutations in the  $\alpha 3$  domain on A2/peptide multimer interaction kinetic. **A**, Binding of wild-type or mutated A2/peptide multimers (1.8  $\mu\text{g}/\text{ml}$ ), incorporating the indicated peptide to CTL clones was assessed after incubation at room temperature for the indicated time period. **B**, Decay of staining over time in the absence or presence of an excess (25  $\mu\text{g}/\text{ml}$ ) of unlabeled multimers incorporating the parental peptide was determined as detailed in *Materials and Methods*. Results are shown as a percentage of maximal staining intensity.

differences are minor compared with those observed in the case of multimers incorporating weak agonists vs parental or full agonist peptides. Together, the results of this study show that efficient simultaneous engagement of CD8 and TCR is required to obtain optimal pMHC multimer binding on specific T cells. This most likely results in the enhancement of T cell function through association of CD8 $\alpha$  with p56<sup>lck</sup> and consequent CD3/TCR phosphorylation. However, the results of this study indicate that decreased pMHC binding to CD8 does not appear to significantly influence pMHC/TCR dissociation rates that remain mostly determined by the TCR and the pMHC ligand.

#### Concluding remarks

Based on previous knowledge of CD8 coreceptor interaction with pMHC, we have used pMHC multimers containing mutations in the  $\alpha 3$  domain known to affect CD8 binding to pMHC to analyze the molecular basis for the decreased binding to specific CD8<sup>+</sup> T

cell clones exhibited by  $\alpha 3$  mutant vs wild-type multimers. At variance with current models, we found that the decreased pMHC interaction with CD8 did not significantly affect pMHC/TCR dissociation kinetics that was dominated by the TCR and the pMHC ligand. Together, our data suggest an alternative model compatible with the most recent data obtained using surface plasmon resonance analysis of molecular interactions between soluble pMHC, TCR, and CD8 $\alpha$  human molecules. According to our model, CD8 binding to pMHC is essential for efficient pMHC/TCR association on the cell surface. Once the pMHC/TCR complex is formed, however, the TCR dominates the interaction with pMHC. This model of function provides a molecular explanation of how the presence of the CD8 coreceptor can efficiently augment the pMHC/TCR interaction in living cells while preserving its specificity. The dynamic coordination of CD8 and TCR binding to pMHC thus contributes the key binding energy that, together with other molecular interactions taking place at the level of the immunological synapse,



orchestrate extra- and intracellular molecular events that rule CD8 T cell activation.

## Acknowledgments

We thank Dr. Donata Rimoldi for kindly providing the tumor cell lines.

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