



Figure 5.

High-throughput screen of functionally relevant tumor-specific CD8⁺ T cells by NTAmers. Relationship between functional avidity and monomeric TCR-pMHC k_{off} rates of a large panel of tumor-specific CD8⁺ T-cell clones specific for A2/NY-ESO-1₁₅₇₋₁₆₅ (A-C) or A2/Melan-A^{MART-1}₂₆₋₃₅ (D-F) tumor antigen and derived from melanoma patient LAU 50 and LAU 618, respectively. A and D, representative first-order dissociation curves obtained after addition of imidazole at 4°C for CD8⁺ A2/NY-ESO-1₁₅₇₋₁₆₅-specific T-cell clones (LAU 50; $n = 67$) and A2/Melan-A^{MART-1}₂₆₋₃₅-specific T-cell clones (LAU 618, $n = 80$), respectively, stained with specific NTAmers and arbitrarily separated into short (white circles) or long (black squares) half-lives according to their $t_{1/2}$ values. B and E, relative functional avidity on a selection of A2/NY-ESO-1₁₅₇₋₁₆₅-specific T-cell clones ($n = 23$) or A2/Melan-A^{MART-1}₂₆₋₃₅-specific T-cell clones ($n = 34$) of short or long half-lives using T2 target cells pulsed with graded concentration of natural NY-ESO-1₁₅₇₋₁₆₅- or Melan-A^{MART-1}₂₆₋₃₅-specific peptide. C and F, positive correlations (Spearman coefficient r and P value) obtained between relative functional avidity by EC_{50} (50% of maximal target cell killing) and monomeric TCR-pMHC dissociation k_{off} values. Each data point represents the result of an individual tumor-specific T-cell clone, averaged from two independent experiments.

(k_{off}) and functional T-cell responses (5, 23–26). Yet, rapid and accurate screening methods to measure TCR-pMHC binding kinetics are still needed to isolate antigen-specific T cells expressing TCRs of high binding avidity. Recently, we developed a novel approach combining reversible peptide-MHC multimers (NTAmers; ref. 15) and real-time flow cytometry (Fig. 1). Using SPR, we had previously determined the TCR-pMHC binding strength of sequence-optimized HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₅-specific TCRs with increasing affinity of up to 150-fold from the wild-type receptor, including two outliers, a very low- and a very high-affinity TCR (5, 20). This TCR panel provides a unique model for validating monomeric TCR-pMHC dissociation kinetics at the surface of T cells by NTAmers. Here, we demonstrate that NTAmer-based off-rates (k_{off} , $t_{1/2}$) followed the same TCR-pMHC binding hierarchy than previously established (20), in excellent agreement to both binding parameters, k_{off} and the dissociation constant K_D , obtained by SPR (Fig. 2; Table 1).

Because of their switch ability, that is, high stability and rapid reversibility (<5 sec), NTAmers allowed accurate determination of dissociation rates, even for weak TCR-pMHC interactions, that is, fast off-rates, such as those found for (self) tumor-specific CD8⁺ T-cell repertoires (Fig. 4). Notably, the NTAmer approach differs from the Streptamer one, which is mostly limited to the detection of CD8⁺ T cells of high avidity such as virus-specific cells, as it requires a significant lag time (60 seconds) before monomeric TCR-pMHC dissociation becomes detectable (11). Moreover, the NTAmer-based assay represents a rapid and straightforward approach for the quantitative assessment of monomeric k_{off} rates on a large set of cloned antigen-specific CD8⁺ T cells derived from different patients and tumor epitopes (HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₅ and HLA-

A*0201/Melan-A^{MART-1}₂₆₋₃₅; Fig. 5). Importantly, we demonstrate robust correlations between dissociation off-rates and the biologic responses (e.g., calcium flux and target cell killing) on a large panel of CD8⁺ T-cell clones specific for two distinct tumor antigens, indicating that TCR-ligand k_{off} rate is a reliable predictor of T-cell function (Figs. 3–5).

Interactions between TCRs and pMHC are usually measured by SPR or pMHC tetramer or multimer staining, which requires one binding partner in soluble form. Both approaches have caveats. Because of their incomplete dissociation and multivalent nature, accurate off-rates data from pMHC tetramer/multimer staining measurements are imprecise. Conversely, monomeric dissociation k_{off} rates measured by the NTAmer technology were within the range of seconds to minutes, spanning a broad range (2-logs), as compared with a narrow range of minutes observed when using pMHC tetramers. Moreover, SPR fails to take into account rapid rebinding of the TCR to the same pMHC, because one of the two binding partners is constantly moving in the fluid phase, which impacts on the binding kinetics. Increased k_{on} rates have been shown to allow rapid rebinding after TCR-ligand dissociation, resulting in enhanced effective dissociation half-life of the TCR-pMHC interaction (25, 27). This may explain our observation that TCR variants with faster k_{on} (e.g., TM α and QM α) showed prolonged NTAmer-based dissociation half-lives compared with soluble monomeric off-rates measured by SPR (Table 1 and Supplementary Fig. S5).

The NTAmer-based approach further deviates from SPR measurements, as it provides data on living cells and includes contributions of CD8 to TCR-pMHC interactions. The CD8 coreceptor enhances antigen recognition and T-cell activation by stabilizing TCR-pMHC interaction at the cell surface

Table 1. Kinetic characteristics of HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₅-specific TCR variants

TCR variants ^a	SPR TCR-pMHC kinetics ^b				NTAmer dissociation kinetics				NTAmer ²²⁷⁻²²⁸ dissociation kinetics			
	Soluble TCRs				CD8 ⁺ ^c		CD8 ⁻ ^c		CD8 ⁺ ^c		CD8 ⁻ ^c	
	K_D $\mu\text{mol/L}$	k_{on} 1/[(mol/L) \times s] ($\times 10^4$)	k_{off} 1/s ($\times 10^{-2}$)	$t_{1/2}$ s	k_{off} 1/s ($\times 10^{-2}$)	$t_{1/2}$ s	k_{off} 1/s ($\times 10^{-2}$)	$t_{1/2}$ s	k_{off} 1/s ($\times 10^{-2}$)	$t_{1/2}$ s	k_{off} 1/s ($\times 10^{-2}$)	$t_{1/2}$ s
V49I	n.a.	n.a.	n.a.	n.a.	21.21	3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Wild-type	21.4	1.1	23.0	3	4.08	17	10.2	7	11.2	6	15.4	5
G50A	4.62	1.5	6.9	10	1.47	47	n.d.	n.d.	5.28	13	n.d.	n.d.
A97L	2.69	2.3	6.1	11	1.60	44	n.d.	n.d.	4.70	15	n.d.	n.d.
DM β	1.91	2.4	4.5	15	0.78	90	2.76	25	3.02	23	3.15	22
TM β	0.91	1.4	1.3	53	0.28	247	1.03	67	0.87	79	0.97	72
TM α	0.40	12.1	4.8	14	0.44	158	n.d.	n.d.	1.76	40	n.d.	n.d.
QM α	0.14	10.9	1.5	46	0.21	341	0.80	87	0.74	94	0.75	92
Wtc51m	0.015	8.5	0.13	533	0.05	1505	0.14	497	0.15	475	0.14	496

Abbreviations: n.a., not applicable; n.d., not done.

^aWild-type TCR (BCI; AV23.1/BV13.1) was isolated from melanoma patient LAU 155 (16) and a panel of TCR variants of progressive increasing affinities against HLA-A*0201/NY-ESO-1₁₅₇₋₁₆₅ was established as described in ref. 20.

^bTCR-pMHC affinity, k_{off} and k_{on} values were previously measured by SPR as reported in ref. 20.

^cCD8⁺ T cells, TCR-transduced SUP-T1 cells; CD8⁻ T cells, TCR-transduced Jurkat cells.

(28–31) and recruiting p56^{lck} to TCR/CD3 complex promoting cell signaling (32, 33). Here, we demonstrate that CD8 strengthened the TCR-pMHC binding mainly by decreasing the TCR-pMHC dissociation by a factor of 3- to 4-fold (Fig. 2), as anticipated by previous tetramer dissociation assays (22, 30). Interestingly, the CD8 stabilization factor was independent of the TCR-pMHC affinity, in contrast with the CD8 dependence for T-cell activation, which can be directly linked to the affinity (3, 20, 22), and allows tuning the sensitivity and specificity of T-cell responses (34).

We recently provided new evidence that T-cell signaling and activation are optimal within a given TCR-pMHC affinity window (20), controlled through TCR affinity-mediated regulatory molecules, involving the inhibitory receptor PD-1 and SHP-1 phosphatase (19). Furthermore, while high-avidity T cells have been shown to control tumor growth, they become preferentially tolerized in the tumor microenvironment (35) or can target normal tissues expressing the cognate antigen (36, 37). Therefore, tumor-specific T cells of high avidity may not always be functionally better, and it remains to be fully determined to which degree intermediate or high-avidity T cells contribute to protective immunity. In this regard, NTAmers constitute a highly valuable tool for assessing the TCR-pMHC avidity and its relation to cell activation, signaling, and function in naturally or therapeutically induced tumor-specific T-cell responses, with TCR-pMHC affinities spanning within the physiologic range.

In summary, NTAmer technology enables efficient and direct interrogation of monomeric TCR-pMHC dissociation kinetics on a large set of living antigen-specific T cells by flow cytometry, and provides novel perspectives for rapid identification of rare functionally relevant tumor-reactive CD8⁺ T cells. Our approach may also be applicable to the analysis of other weak protein-protein interactions. Precise and widespread characterization of TCR-

pMHC avidities will likely improve the development of T-cell-based immunotherapies in patients with cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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