Published OnlineFirst March 25, 2015; DOI: 10.1158/0008-5472.CAN-14-3516

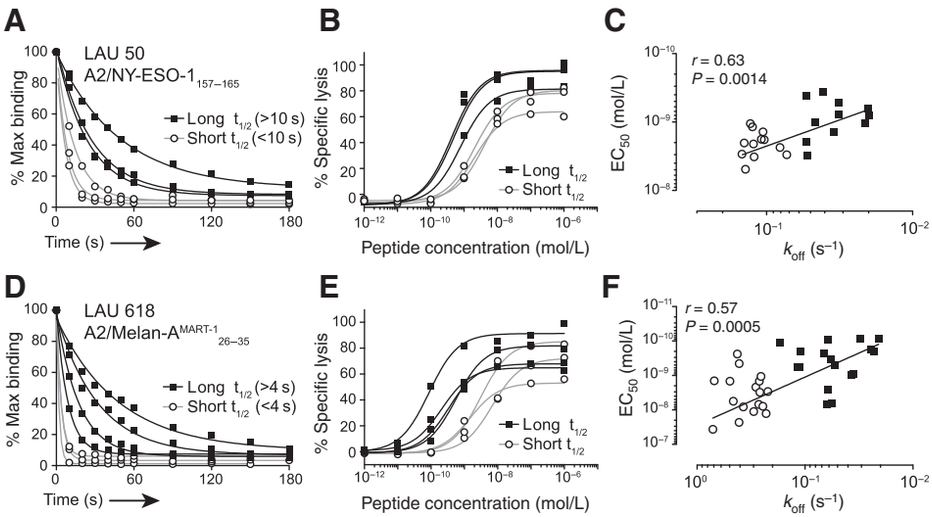

Figure 5.

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-1$_{157–165}$ (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-1$_{157–165}$–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-1$_{157–165}$–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-1$_{157–165}$– 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-1$_{157–165}$–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 *Strep*tamer 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-1$_{157–165}$ 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., TMα and QMα) 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

Published OnlineFirst March 25, 2015; DOI: 10.1158/0008-5472.CAN-14-3516

Table 1. Kinetic characteristics of HLA-A*0201/NY-ESO-$1_{157-165}$–specific TCR variants

	SPR TCR–pMHC kinetics[b] Soluble TCRs				NTAmer dissociation kinetics				$NTAmer^{227-228}$ dissociation kinetics			
					$CD8^{+c}$		$CD8^{-c}$		$CD8^{+c}$		$CD8^{-c}$	
TCR variants[a]	K_D μmol/L	k_{on} 1/[(mol/L)× s] (×10^4)	k_{off} 1/s (×10^{-2})	$t_{1/2}$ s	k_{off} 1/s (×10^{-2})	$t_{1/2}$ s	k_{off} 1/s (×10^{-2})	$t_{1/2}$ s	k_{off} 1/s (×10^{-2})	$t_{1/2}$ s	k_{off} 1/s (×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.
DMβ	1.91	2.4	4.5	15	0.78	90	2.76	25	3.02	23	3.15	22
TMβ	0.91	1.4	1.3	53	0.28	247	1.03	67	0.87	79	0.97	72
TMα	0.40	12.1	4.8	14	0.44	158	n.d.	n.d.	1.76	40	n.d.	n.d.
QMα	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.

[a]Wild-type TCR (BC1; 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-$1_{157-165}$ was established as described in ref. 20.

[b]TCR–pMHC affinity, k_{off} and k_{on} values were previously measured by SPR as reported in ref. 20.

[c]$CD8^+$ 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.

Authors' Contributions

Conception and design: M. Hebeisen, J. Schmidt, D.E. Speiser, N. Rufer
Development of methodology: M. Hebeisen, J. Schmidt, P. Guillaume, D.E. Speiser
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Hebeisen, J. Schmidt, P. Baumgaertner, N. Rufer
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Hebeisen, N. Rufer
Writing, review, and/or revision of the manuscript: M. Hebeisen, J. Schmidt, D.E. Speiser, N. Rufer
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.E. Speiser, I. Luescher
Study supervision: N. Rufer

Acknowledgments

The authors thank P. Gannon, M. Irving, O. Michielin, P. Romero, S. Siegert, and L. Zhang for essential collaboration and advice, and N. Montandon and M. van Overloop for outstanding technical assistance.

Grant Support

This work was supported by the Department of Oncology of the University of Lausanne and the Ludwig Center for Cancer Research of the University of Lausanne.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 29, 2014; revised February 3, 2015; accepted February 20, 2015; published OnlineFirst March 25, 2015.

References

1. Stone JD, Kranz DM. Role of T cell receptor affinity in the efficacy and specificity of adoptive T cell therapies. Front Immunol 2013;4:244.
2. Zeh HJ III, Perry-Lalley D, Dudley ME, Rosenberg SA, Yang JC. High avidity CTLs for two self-antigens demonstrate superior *in vitro* and *in vivo* antitumor efficacy. J Immunol 1999;162:989–94.
3. Holler PD, Kranz DM. Quantitative analysis of the contribution of TCR/pepMHC affinity and CD8 to T cell activation. Immunity 2003;18:255–64.
4. Tian S, Maile R, Collins EJ, Frelinger JA. CD8+ T cell activation is governed by TCR–peptide/MHC affinity, not dissociation rate. J Immunol 2007;179:2952–60.

5. Schmid DA, Irving MB, Posevitz V, Hebeisen M, Posevitz-Fejfar A, Sarria JC, et al. Evidence for a TCR affinity threshold delimiting maximal CD8 T cell function. J Immunol 2010;184:4936–46.
6. Foley MH, Forcier T, McAndrew E, Gonzalez M, Chen H, Juelg B, et al. High avidity $CD8^+$ T cells efficiently eliminate motile HIV-infected targets and execute a locally focused program of anti-viral function. PLoS ONE 2014;9: e87873.
7. Tan MP, Gerry AB, Brewer JE, Melchiori L, Bridgeman JS, Bennett AD, et al. TCR binding affinity governs the functional profile of cancer-specific CD8 T cells. Clin Exp Immunol 2014 Dec 15. [Epub ahead of print].
8. Aleksic M, Liddy N, Molloy PE, Pumphrey N, Vuidepot A, Chang KM, et al. Different affinity windows for virus and cancer-specific T-cell receptors: implications for therapeutic strategies. Eur J Immunol 2012;42:3174–9.
9. Wang XL, Altman JD. Caveats in the design of MHC class I tetramer/antigen-specific T lymphocytes dissociation assays. J Immunol Methods 2003;280:25–35.
10. Schmidt J, Dojcinovic D, Guillaume P, Luescher I. Analysis, isolation, and activation of antigen-specific CD4(+) and CD8(+) T cells by soluble MHC-peptide complexes. Front Immunol 2013;4:218.
11. Nauerth M, Weissbrich B, Knall R, Franz T, Dossinger G, Bet J, et al. TCR–ligand koff rate correlates with the protective capacity of antigen-specific CD8+ T cells for adoptive transfer. Sci Transl Med 2013;5:192ra87.
12. Huang J, Zarnitsyna VI, Liu B, Edwards LJ, Jiang N, Evavold BD, et al. The kinetics of two-dimensional TCR and pMHC interactions determine T-cell responsiveness. Nature 2010;464:932–6.
13. Huppa JB, Axmann M, Mortelmaier MA, Lillemeier BF, Newell EW, Brameshuber M, et al. TCR–peptide–MHC interactions in situ show accelerated kinetics and increased affinity. Nature 2010;463:963–7.
14. Liu B, Zhong S, Malecek K, Johnson LA, Rosenberg SA, Zhu C, et al. 2D TCR–pMHC–CD8 kinetics determines T-cell responses in a self-antigen-specific TCR system. Eur J Immunol 2014;44:239–50.
15. Schmidt J, Guillaume P, Irving M, Baumgaertner P, Speiser D, Luescher IF. Reversible major histocompatibility complex I-peptide multimers containing Ni(2+)-nitrilotriacetic acid peptides and histidine tags improve analysis and sorting of CD8(+) T cells. J Biol Chem 2011;286:41723–35.
16. Derre L, Bruyninx M, Baumgaertner P, Ferber M, Schmid D, Leimgruber A, et al. Distinct sets of alphabeta TCRs confer similar recognition of tumor antigen NY-ESO-1157-165 by interacting with its central Met/Trp residues. Proc Natl Acad Sci U S A 2008;105:15010–5.
17. Purbhoo MA, Boulter JM, Price DA, Vuidepot AL, Hourigan CS, Dunbar PR, et al. The human CD8 coreceptor effects cytotoxic T cell activation and antigen sensitivity primarily by mediating complete phosphorylation of the T cell receptor zeta chain. J Biol Chem 2001;276:32786–92.
18. Zoete V, Irving M, Ferber M, Cuendet MA, Michielin O. Structure-based, rational design of T cell receptors. Front Immunol 2013;4:268.
19. Hebeisen M, Baitsch L, Presotto D, Baumgaertner P, Romero P, Michielin O, et al. SHP-1 phosphatase activity counteracts increased T cell receptor affinity. J Clin Invest 2013;123:1044–56.
20. Irving M, Zoete V, Hebeisen M, Schmid D, Baumgartner P, Guillaume P, et al. Interplay between T cell receptor binding kinetics and the level of cognate peptide presented by major histocompatibility complexes governs CD8+ T cell responsiveness. J Biol Chem 2012;287:23068–78.
21. Holler PD, Chlewicki LK, Kranz DM. TCRs with high affinity for foreign pMHC show self-reactivity. Nat Immunol 2003;4:55–62.
22. Laugel B, van den Berg HA, Gostick E, Cole DK, Wooldridge L, Boulter J, et al. Different T cell receptor affinity thresholds and CD8 coreceptor dependence govern cytotoxic T lymphocyte activation and tetramer binding properties. J Biol Chem 2007;282:23799–810.
23. Dutoit V, Rubio-Godoy V, Doucey MA, Batard P, Lienard D, Rimoldi D, et al. Functional avidity of tumor antigen-specific CTL recognition directly correlates with the stability of MHC/peptide multimer binding to TCR. J Immunol 2002;168:1167–71.
24. La Gruta NL, Doherty PC, Turner SJ. A correlation between function and selected measures of T cell avidity in influenza virus-specific CD8+ T cell responses. Eur J Immunol 2006;36:2951–9.
25. Aleksic M, Dushek O, Zhang H, Shenderov E, Chen JL, Cerundolo V, et al. Dependence of T cell antigen recognition on T cell receptor-peptide MHC confinement time. Immunity 2010;32:163–74.
26. Dushek O, Aleksic M, Wheeler RJ, Zhang H, Cordoba SP, Peng YC, et al. Antigen potency and maximal efficacy reveal a mechanism of efficient T cell activation. Sci Signal 2011;4:ra39.
27. Govern CC, Paczosa MK, Chakraborty AK, Huseby ES. Fast on-rates allow short dwell time ligands to activate T cells. Proc Natl Acad Sci U S A 2010;107:8724–9.
28. Luescher IF, Vivier E, Layer A, Mahiou J, Godeau F, Malissen B, et al. CD8 modulation of T-cell antigen receptor-ligand interactions on living cytotoxic T lymphocytes. Nature 1995;373:353–6.
29. Gao GF, Tormo J, Gerth UC, Wyer JR, McMichael AJ, Stuart DI, et al. Crystal structure of the complex between human CD8alpha(alpha) and HLA-A2. Nature 1997;387:630–4.
30. Wooldridge L, van den Berg HA, Glick M, Gostick E, Laugel B, Hutchinson SL, et al. Interaction between the CD8 coreceptor and major histocompatibility complex class I stabilizes T cell receptor-antigen complexes at the cell surface. J Biol Chem 2005;280: 27491–501.
31. Puech PH, Nevoltris D, Robert P, Limozin L, Boyer C, Bongrand P. Force measurements of TCR/pMHC recognition at T cell surface. PLoS ONE 2011;6:e22344.
32. Artyomov MN, Lis M, Devadas S, Davis MM, Chakraborty AK. CD4 and CD8 binding to MHC molecules primarily acts to enhance Lck delivery. Proc Natl Acad Sci U S A 2010;107:16916–21.
33. Jiang N, Huang J, Edwards LJ, Liu B, Zhang Y, Beal CD, et al. Two-stage cooperative T cell receptor-peptide major histocompatibility complex-CD8 trimolecular interactions amplify antigen discrimination. Immunity 2011;34:13–23.
34. Cole DK, Laugel B, Clement M, Price DA, Wooldridge L, Sewell AK. The molecular determinants of CD8 co-receptor function. Immunology 2012; 137:139–48.
35. Zhu Z, Singh V, Watkins SK, Bronte V, Shoe JL, Feigenbaum L, et al. High-avidity T cells are preferentially tolerized in the tumor microenvironment. Cancer Res 2013;73:595–604.
36. Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood 2009;114:535–46.
37. Morgan RA, Chinnasamy N, Abate-Daga D, Gros A, Robbins PF, Zheng Z, et al. Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. J Immunother 2013;36:133–51.