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Quantitative TCR:pMHC Dissociation Rate Assessment by NTAmers Reveals Antimelanoma T Cell Repertoires Enriched for High Functional Competence

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Experimental models demonstrated that therapeutic induction of CD8 T cell responses may offer protection against tumors or infectious diseases providing that T cells have sufficiently high TCR/CD8:pMHC avidity for efficient Ag recognition and consequently strong immune functions. However, comprehensive characterization of TCR/CD8:pMHC avidity in clinically relevant situations has remained elusive. In this study, using the novel NTA-His tag–containing multimer technology, we quantified the TCR:pMHC dissociation rates ($k_{\text{off}}$) of tumor-specific vaccine-induced CD8 T cell clones ($n = 139$) derived from seven melanoma patients vaccinated with IFA, CpG, and the native/EAA or analog/ELA Melan-A MART-1^26-35 Peptide, binding with low or high affinity to MHC, respectively. We observed substantial correlations between $k_{\text{off}}$ and Ca^{2+} mobilization ($p = 0.016$) and target cell recognition ($p < 0.0001$), with the latter independently of the T cell differentiation state. Our strategy was successful in demonstrating that the type of peptide impacted on TCR/CD8:pMHC avidity, as tumor-reactive T cell clones derived from patients vaccinated with the low-affinity (native) peptide expressed slower $k_{\text{off}}$ rates than those derived from patients vaccinated with the high-affinity (analog) peptide ($p < 0.0001$). Furthermore, we observed that the low-affinity peptide promoted the selective differentiation of tumor-specific T cells bearing TCRs with high TCR/CD8:pMHC avidity ($p < 0.0001$). Altogether, TCR:pMHC interaction kinetics correlated strongly with T cell functions. Our study demonstrates the feasibility and usefulness of TCR/CD8:pMHC avidity assessment by NTA-His tag–containing multimers of naturally occurring polyclonal T cell responses, which represents a strong asset for the development of immunotherapy.

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P roductive T cell responses are characterized by strong initial clonal T cell bursts arising from a relatively small number of Ag-specific naive precursors. Following the encounter with their cognate peptide presented by MHC (pMHC), T cells proliferate and acquire various cellular survival and effector functions assuring long-term persistence, homing to inflamed tissues, and cytotoxic capacities. T cells can differentiate into memory and effector cells, with the extent of T cell differentiation being dependent, in part, on the strength of signals emanating from the TCR (1). Compared with other molecular interactions, the TCR:pMHC-binding strength is relatively weak (∼1–100 μM) with fast dissociation kinetics ($t_{1/2} < 60$ s) (2). TCR:pMHC interactions can be measured in terms of affinity or avidity, both of which can directly impact the overall functional T cell response (e.g., cytokine secretion, target cell killing) (3). The physical strength with which a TCR binds to a given pMHC complex is referred as the affinity of the TCR:pMHC interaction. In contrast, in the cellular context, TCR/CD8:pMHC avidity defines the strength of binding of monomeric TCR on the pMHC with the inclusion of CD8 co-receptor binding.

In cancer, CD8 T cells often recognize tumor-associated Ags of self-origins, and, due to thymic negative selection, tumor-specific T cells express TCRs of lower affinity/avidity compared with TCRs specific for viral, nonself epitopes, or mutated epitopes (4). Consequently, there is an expanding body of work on engineering TCR:pMHC binding kinetics have yet to be considered as a determining factor in the selection of Ag-specific T cells for clinical protocols. Furthermore, although the TCR:pMHC affinity/avidity for pMHC is a major determinant of thymic selection (10), autoimmune pathogenicity (11–13), and T cell responsiveness (2), the precise impact of TCR:pMHC-binding parameters ($k_{\text{on}}$ and $k_{\text{off}}$) on CD8 T cell repertoire selection and differentiation within the periphery in well-defined clinical settings, such as therapeutic vaccination, remains to be directly evaluated. To this day, a debate remains regarding the accurate method to measure TCR:pMHC-binding kinetics ($k_{\text{on}}$ and $k_{\text{off}}$) and which

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parameter(s) could better predict T cell activation and function (14). Whereas pMHC multimers were often used in binding and dissociation assays, results are biased by the multivalent nature of multimers and the ensuing use of rebinding antagonists (15, 16). Conversely, three-dimensional soluble TCR:pMHC affinity measurements by surface plasmon resonance (SPR) omit the impact of CD8 coreceptor binding and membrane fluidity (15). The two-dimensional techniques have offered important membrane-associated kinetics insights (17) and led to the elaboration of mathematical models (14) predicting TCR:pMHC interactions and their association with T cell functions. However, these labor-intensive techniques are not well suited for the high-throughput characterization of living Ag-specific T cells. Recently, novel approaches have allowed the direct and precise quantification of TCR:pMHC dissociation rates ($k_{off}$) on living CD8 T cells by real-time flow cytometry (15, 18) or microscopy (19). Among these, the reversible NTA-His tag–containing multimers (NTAmers), which can be rapidly dissociated into monomeric pMHC (15, 18), allow the accurate measurements of dissociation rates ($k_{off}$), even for weak TCR:pMHC interactions, such as those found in tumor-specific CD8 T cell repertoires (20). This technique allows for the quantification of dissociation kinetics ($k_{off}$) with higher sensitivity than multimer-based assays (20). Importantly, the monomeric dissociations of NTAmers account for the impact of the CD8 coreceptor in the overall avidity of the TCR for its cognate pMHC, an aspect not taken into consideration during SPR experiments and defined thereafter as TCR/CD8:pMHC avidity. Nevertheless, the $k_{off}$ values measured by NTAmers correlate significantly to those derived from SPR assays (20). Together, the NTamer technology represents a powerful tool to precisely quantify TCR:pMHC dissociation rates ($k_{off}$) on living CD8 T cells by real-time flow cytometry and at monomeric resolution. Most groups use transgenically modified TCR or altered peptide libraries to establish an affinity range on which one can then evaluate the impact of TCR/CD8:pMHC avidity on T cell activation and functionality. Few studies are based on quantifying the association between TCR/CD8:pMHC avidity and T cell responsiveness in naturally occurring TCR repertoires and in humans. Our group has thoroughly characterized a cohort of melanoma patients who received peptide-based vaccination in combination with CpG and IFN (21). Interestingly, patients vaccinated with the native HLA-A2/Melan-A$^{26–35}$ peptide developed antitumor T cell responses with improved functionality compared with patients vaccinated with the analog Melan-A$^{26–35}$ (A27L) Peptide (22–24). We will define hereafter the analog peptide as high affinity in comparison with the low-affinity native peptide as the analog Melan-A$^{26–35}$ (A27L) Peptide has a 10-fold increased binding to HLA-A2 (25). The vaccine-induced tumor-specific CD8 T cell clones derived from these patients thus represent valuable tools to inquire the relationship between TCR:pMHC kinetics, T cell repertoire selection, and functionality in a clinically relevant cancer setting.

In this study, we show that the use of a low-affinity (native) peptide in a therapeutic vaccination setting of melanoma patients favored the selection/differentiation of tumor Ag-specific T cells bearing TCRs with high TCR/CD8:pMHC avidity. Using a large series of Melan-A$^{26–35}$–specific clones ($n = 92$), we demonstrate that CD8 T cells with slower dissociation rates ($k_{off}$) and longer $t_{1/2}$ had improved functional avidity (Ca$^{2+}$ flux and killing potential). Together, our work shows the feasibility of TCR/CD8:pMHC avidity assessment in patients using NTAmers and offers novel indications that low-affinity (native peptide) vaccination increases anti-cancer TCR/CD8:pMHC avidity and CD8 T cell functionality.
TCR β-chain repertoire and clonotype analysis. cDNA preparation and CDR3 spectratyping, sequencing, and clonotyping were performed, as described (24, 26). Briefly, each cDNA sample was subjected to individual PCR using a set of previously validated fluorescent-labeled forward primers specific for the 22 TRBV subfamilies and one unlabeled reverse primer specific for the corresponding Cβ gene segment. Additionally, we characterized the α-chain repertoire by targeting the highly dominant TRAV12-1 sequence. PCR products of interest were sequenced from the reverse primer and TRBV segments were recorded and analyzed according to the Lefranc nomenclature (29). Term of “dominant clonotype” refers to a nucleotide sequence found with a frequency >5% within a given patient.

Chromium release cytolytic assays. Chromium release cytolytic assays were performed, as previously described (28). Briefly, 51Cr-labeled TAP dually labeled EAA Melan-AMART-1 deficient T2 (HLA-A*0201+ Melan-A) lysis. Nonkiller clones were defined as clones presenting a maximal lysis according to the Lefranc nomenclature (29). Term of “dominant clono-

Calcium mobilization assays. Calcium mobilization assays were performed as previously described (30). Briefly, after loading with 2 μM Indo 1-AM (Sigma-Aldrich), baseline signal was recorded for 30 s before 1 μM of the native or analog Melan-A M1-26–35 peptide with an E:T ratio of 10:1. The melanoma cell lines Me290 and NA8 were also used as targets in the absence or presence of the analog Melan-A M1-26–35 peptide (1 μM) at an E:T ratio of 30:1 (23). An irrelevant Flu peptide (MA, 1 μM) and a no-peptide control were used as negative controls.

The percentage of specific lysis was calculated as 100 X (experimental – spontaneous release)/total – spontaneous release). The EC50 value was de-

NTAmer staining and dissociation kinetic measurements. Dually labeled pMHC multimers built on NTA-Ni2+-His-tag interactions called NTAmers were used for dissociation kinetic measurements and synthesized by TCMatrix Sarl, as described previously (15, 18). Stainings with 8 nM NTAmer technology is depicted in Supplemental Fig. 1C.

Data analysis Statistical analyses. Data were analyzed using GraphPad Prism software (v.5). Student's t test as well as nonparametric Mann-Whitney, Wilcoxon paired t test, Kruskal-Wallis, and Spearman’s correlations, as indicated throughout the manuscript. For all experiments, raw data can be provided upon request.

Laboratory environment. These studies were conducted in a laboratory that operates under Good Laboratory Practices principles. The laboratory participates in external ELISPOT and immune-monitoring proficiency panels. The study was performed using established laboratory protocols. These studies were performed using a qualified assay.

Results Functional efficiency of tumor-specific T clones is primarily determined by the type of peptide used for vaccination. In previous publications in which Melan A–specific CD8 T cells were analyzed directly ex vivo, we have demonstrated an im-

affinity (analog) peptide. These results were based on various assays including ex vivo single cell gene expression profiling (23, 24), ex vivo intracellular cytokine staining (22, 23), ex vivo IFN-γ ELISPOT (22, 24), and 51Cr-release cytotoxicity using T2-pulsed target (22, 24) or melanoma cell lines (23). Overall, these data on polyclonal Melan-A–specific CD8+ T cell populations provided convincing results demonstrating the superior antitumoral immune response generated by vaccination with the low-affinity (native) peptide formulation.

For this study, we generated a large library of T cell clones (n = 150) bearing well-defined TCR-αβ clonotypes isolated from melanoma patients vaccinated with either the low-affinity (native/ EAA, n = 3) or the high-affinity (analog/ELA, n = 4) peptides, and derived from both the early and late differentiated subsets (defined as EM multimer+/CD8+/CD45RA- /CCR7- /CD28+ [EM8]+ and CD28+ [EM8−], respectively). To validate this collection of tumor Ag-specific T cell clones with the previously gathered ex vivo data (22–24), we first assessed their functional efficiency in terms of target cell killing, expression of effector molecules, and Ca2+ mobilization capacity.

Extending on previous reports (23, 24), we observed that EM28+ and EM28− T cell clones of low-affinity (native) peptide-vaccinated patients had a 10-fold increase in functional avidity compared with killing responses obtained from the corresponding subsets derived from high-affinity (analog) peptide-vaccinated patients (p = 0.002 and p < 0.001, respectively) (Fig. 1A, left panel). We defined functional avidity in relation with the EC50 (50% maximal lysis effective peptide concentration) of T cell clones from the killing assays. Moreover, in contrast to analog ELA/EM28+ tumor-specific T cell clones showing a significant fraction of clones with poor cytolytic activity (defined as non-
killers), almost all native EAA/EM28+ clones were able to lyse peptide-pulsed target cells (Fig. 1A, right panel). Abrogating CD8 binding to pMHC by using target cells transfected with D227K/ T228A HLA-A2 mutant led to a >10-fold decrease in target recognition capacity for both EAA and ELA clones (p < 0.001 and p = 0.006, respectively) (Fig. 1B, left panel) with an increased frequency of nonkiller ELA clones (Fig. 1B, right panel). Importantly, these functional avidity differences between tumor-specific CD8 T cell clones derived from the two cohorts of patients were discernable only when the low-affinity (native) peptide was used during the in vitro stimulation. Due to its 10-fold higher HLAA2–binding affinity (25), the high-affinity (analog) peptide stimulation yielded comparable EC50 values between both vaccination cohorts (Supplemental Fig. 2A, 2B, left panel). We also evaluated the tumor reactivity of the CD8 T cell clones for melanoma tumor cell lines. Against the HLAA2+ Me290 cell line expressing the endogenous Melan-A Ag, the EAA clones in both EM28+ and EM28− subsets had a slightly higher killing efficiency than their ELA counterparts, with again a large proportion of ELA-derived T cell clones that were deficient in cytotoxic func-

tion and were thus defined as nonkillers (Fig. 1C, left panel). The same assay using the HLAA2−/Melan-A-deficient NA8 cell line demonstrated the Ag specificity of the selected clones, as most clones were unable to recognize and kill these target cells (Fig. 1D). By pulsing the Me290 or the NA8 cell lines with the analog ELA peptide, we again could observe a higher killing efficiency for the EAA-derived clones (Fig. 1C, 1D, right panels).

We then assessed whether tumor-specific CD8 T cells from low-

affinity (native) peptide-vaccinated patients had qualitative biological differences promoting their increased killing capacity. Clones showed similar maximum Ca2+ fluxing potential (Fig. 1E, left panel) independently of the subset (EM28+ versus EM28−) or vaccination cohort (native versus analog). Nonetheless, we continued
FIGURE 1. Functional competence of tumor Ag-specific CD8 T cell clones derived following low (native)- or high (analog)-affinity Melan-A peptide vaccination. (A) EC50 values (50% of maximum lysis) (left panel) from cytolytic assays performed with chromium-labeled Melan-A-negative T2 target cells loaded with graded concentrations of low-affinity (native) Melan-A\textsuperscript{26–35} peptide. Tumor-specific T cell clones were derived from seven vaccinated melanoma patients (native/EAA, n = 78 and analog/ELA, n = 72). Frequency of nonkiller clones (right panel) with low cytolytic activity from which EC50 values could not be accurately calculated (i.e., as the percentage of maximum lysis <25%). The p values are by Kruskal–Wallis. (B) EC50 values (left panel) from cytolytic assays performed using C1R cells transfected with either the wild-type (WT) (n = 100) or HLA-A2 molecules deficient for CD8 binding (Mut) (n = 98) and loaded with graded concentrations of low-affinity (native) Melan-A\textsuperscript{26–35} peptide. EM28\textsuperscript{+} and EM28\textsuperscript{−} T cell clones were pooled together. Frequency of nonkiller clones (right panel). The p values are by Wilcoxon paired t test. (C and D) Tumor reactivity for melanoma cell lines Me290 (HLA-A2+/Melan-A\textsuperscript{+}) (n = 80) (C) and NA8 (HLA-A2+/Melan-A\textsuperscript{−}) (n = 87) (D) at an E/T ratio of 30:1 in the presence (right panels) or absence (left panels) of analog/ELA peptide (1 μM). The p values are by Kruskal–Wallis. Nonkiller clones were defined as clones with a maximum lysis <25%. Of note, the majority of EAA- and ELA-specific clones were unable to efficiently kill the control NA8 tumor cell line, when no exogenous Melan-A peptide was added to the assay. (E) Ca\textsuperscript{2+} flux (left panel) of CD8 T cell clones (n = 95) following 1 μg/ml HLA-A2 low-affinity (native) Melan-A\textsuperscript{26–35} peptide stimulation. Data expressed as peak mean fluorescence intensity (MFI) from the Indo-1 (violet)/Indo-1 (blue) emission ratio. Frequency of tumor-specific T cell clones without detectable Ca\textsuperscript{2+} flux (right panel). The p values are by Kruskal–Wallis. (F) Summary of all MFI measured in intracellular staining experiments for granzyme B (left panel) and perforin (right panel) on unstimulated tumor-specific CD8 T cell clones (n = 63). The p values are by Kruskal–Wallis. (A–F) Clones were classified according to subset (EM28\textsuperscript{+} versus EM28\textsuperscript{−}) and peptide vaccination (native/EAA versus analog/ELA). Box (25th and 75th percentile) and whisker (5th and 95th percentile) graph with the middle line representing the median. EAA clones (green), ELA clones (blue), EM28\textsuperscript{+} clones (○), and EM28\textsuperscript{−} clones (□). The n values represent the sum of the killer and nonkiller clones.
to find unresponsive tumor-specific T cell clones within the ELA cohort and a higher prevalence of clones with deficient Ca\(^{2+}\) flux within both ELA/EM28\(^{+}\) and ELA/EM28\(^{-}\)-derived clones (Fig. 1E, right panel). By intracellular staining, we observed that the EAA/EM28\(^{+}\) clones expressed significantly more granzyme B and perforin than their ELA/EM28\(^{+}\) counterparts at resting state (Fig. 1F).

Together, the data obtained from this large collection of vaccine-induced, tumor-specific CD8\(^{+}\) T cell clones extended on our recent ex vivo analyses (23, 24) by showing that the type of peptide vaccine (low or high affinity) qualitatively impacted the T cell cytotoxicity, the expression of effector molecules, to a lesser extent Ca\(^{2+}\) signaling, and with significant differences observed in the frequencies of nonfunctional clones (nonkiller or non-Ca\(^{2+}\) flux).

**Functional efficiency is independent of clonotype frequency**

The TCR repertoire of the tumor-specific CD8\(^{+}\) T cell clone library was, within the early differentiated EM28\(^{+}\) subset, predominantly composed of individual and nondominant clonotypes, which contrasted with the preferential selection of many codominant clonotypes in the differentiated EM28\(^{-}\) subset (Supplemental Fig. 3). In line with our previous report (23), we observed that the functional killing avidity, as defined by EC\(_{50}\) values, was independent of clonotype frequency and instead was associated with the vaccination cohort (Fig. 2A). To exclude the potential bias due to the heterogeneous nature of the clonotype repertoire among T cell subsets, we further tested the functional avidity of EM28\(^{+}\) and EM28\(^{-}\) T cell clones bearing the identical TCR (same TRA V and TRBV sequences) (Fig. 2B, Supplemental Fig. 2C). The TRBV19-1 clones from patient LAU 618, vaccinated with the high-affinity (analog) peptide, showed decreased functional avidity with a higher EC\(_{50}\) than the TRBV6c1 clones from patient LAU 1013 vaccinated with the low-affinity (native) peptide vaccination. Moreover, the EM28\(^{+}\) TRBV19c1 clones from LAU 618 had lower maximum lysis potential than their EM28\(^{-}\) counterparts (Fig. 2B, right panel), contrasting to the similar maximum lysis found for both subsets from LAU 1013 (Fig. 2B, left panel). Together, the functional heterogeneity present in our large clonal library yielded similar results to our previous ex vivo analyses based on the same cohorts of patients (22–24, 31), thereby indicating that these clones provide a powerful model to study the impact of TCR/CD8:pMHC avidity on vaccine-induced tumor-specific T cell responses in a clinically relevant setting.

**Repeated vaccination with low-affinity peptide favors the differentiation of tumor-specific CD8 T cells with high TCR/CD8:pMHC avidity TCRs**

To gain insight into the TCR/CD8-binding avidity for pMHC, we relied on a novel technology based on dual-color reversible multimers composed of multivalent NTAmer, which can be used to quantitatively assess TCR:pMHC dissociation (\(k_{\text{off}}\)) rates on living T cells (15, 18, 20). Following the addition of nontoxic low concentration of imidazole, the multimers decayed into Cy5-labeled pMHC monomers and PE-labeled streptavidin NTA4 molecules within seconds (Fig. 3, Supplemental Fig. 1C). The Cy5 signal from the pMHC complexes then allowed for the accurate measurement of pMHC dissociation kinetics on live CD8 T cells directly by real-time flow cytometry and at monomeric resolution. Using the sensitive NTAmer technology on our tumor-specific

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Functional avidity according to the clonal frequency of Melan-A–specific CD8 T cell clones expressing defined TRBV clonotypes. (A) EC\(_{50}\) values from cytolytic assays with low-affinity (native) Melan-A\(^{\text{MART-1}}\)\(_{26-35}\) peptide stimulation (\(y\)-axis) in relationship with individual T cell clonotype frequency (\(x\)-axis) for tumor-specific CD8 clones from 40 individual TCR clonotypes derived following low-affinity (native, left panel) and high-affinity (analog, right panel) peptide vaccination. Nonkiller clones are depicted in the gray upper box. Mean EC\(_{50}\) value (continuous line) ± 1 SD (dotted lines) for the respective low-affinity (native) and high-affinity (analog) peptide vaccination cohorts. Each symbol represents the average EC\(_{50}\) values of tumor-specific T cell clones bearing the identical TCR (same TRAV and TRBV sequences) (Fig. 2B, Supplemental Fig. 2C). The TRBV19-1 clones from patient LAU 618, vaccinated with the high-affinity (analog) peptide, showed decreased functional avidity with a higher EC\(_{50}\) than the TRBV6c1 clones from patient LAU 1013 vaccinated with the low-affinity (native) peptide vaccination. Moreover, the EM28\(^{+}\) TRBV19c1 clones from LAU 618 had lower maximum lysis potential than their EM28\(^{-}\) counterparts (Fig. 2B, right panel), contrasting to the similar maximum lysis found for both subsets from LAU 1013 (Fig. 2B, left panel).
CD8 T cell clone library, we were able to reliably quantify the dissociation kinetics ($k_{\text{off}}$) of TCR:pMHC complexes along a relatively wide range ($t_{1/2}$ from 0.22 to 974 s), which most probably correlates with a broad spectrum of TCR affinity given their biophysical interdependency ($K_D = k_{\text{on}}/k_{\text{off}}$) of naturally occurring physiological TCRs. Importantly, we used NTAmers loaded with the low-affinity (native) Melan-A peptide as they provided a more physiological assessment of the TCR:pMHC recognition efficacy (Fig. 1, Supplemental Fig. 2).

We observed that the type of peptide used for vaccination could impact the TCR/CD8:pMHC avidity of Ag-specific T cells. The EAA clones had significantly slower dissociation rates than the ELA clones ($k_{\text{off}}$ of 0.041 s$^{-1}$ and 0.130 s$^{-1}$, respectively, $p < 0.0001$). We did not detect any significant difference between EM28$^+$ and EM28$^-$ clones when pooling all T cell clones generated from both vaccination cohorts ($k_{\text{off}}$ of 0.092 s$^{-1}$ and 0.120 s$^{-1}$, respectively, $p > 0.05$) (Fig. 4A, left panel). We then characterized $k_{\text{off}}$ rates according to subset and peptide used during the vaccination. For the early differentiated EM28$^+$ clones, we observed a tendency toward slower dissociation rates within the EAA/EM28$^+$ clones compared with ELA/EM28$^+$ clones ($k_{\text{off}}$ of 0.052 s$^{-1}$ and 0.111 s$^{-1}$, respectively, $p = 0.100$) (Fig. 4A, right panel). Importantly, these slower dissociation rates were more prevalent within the differentiated EAA/EM28$^+$ clones than in their ELA/EM28$^+$ counterparts ($k_{\text{off}}$ of 0.020 s$^{-1}$ versus 0.155 s$^{-1}$, respectively, $p < 0.0001$). Similar differences were obtained when using the NTamer $t_{1/2}$ values instead of the $k_{\text{off}}$ rates (Supplemental Fig. 4).

By classifying the tumor-specific T cell clones according to their respective patient, we observed a higher variance in patients vaccinated with the high-affinity (analog) peptide ($p < 0.0001$, F-test), with the exception of patient LAU 444 (Fig. 4B). This particular patient was studied in detail by our group (32) and had a pre-existing and persistent clonotype (TRBV6c2, also named “ELGTASY”), which dominated the differentiated EM28$^+$ subset and was boosted efficiently after vaccination. We further witnessed the robustness of the NTamer technique through the analysis of tumor-specific T cell clones bearing the same TCR clonotype (identical TRAV and TRBV sequences), which displayed highly similar $k_{\text{off}}$ rates (Fig. 4C). Together, these results indicated that the low-affinity (native) peptide vaccination promoted
together, the NTAmber-based approach for the monomeric TCR: pMHC dissociation constant (k_{off}) rates from T cell clones (n = 139) derived from three low-affinity (native) (LAU 972, LAU 1013, and LAU 1015) and four high-affinity (analog) (LAU 444, LAU 618, LAU 672, and LAU 936) vaccinated patients and depicted (left panel) in terms of total EAA or ELA clones or pooled EM28± and EM28± clones. The p values are by Mann–Whitney. k_{off} rates (right panel) based on the clonal differentiation (EM28± versus EM28±) and peptide used during vaccination (native versus analog). The p values are by Kruskal–Wallis. Box (25th and 75th percentile) and whisker (5th and 95th percentile) graph with the middle line representing the median. Note that there is a statistically significant difference between the EAA/EM28± and EAA/EM28 α clones. (B) k_{off} values categorized according to the respective patients and peptide vaccination cohort (n = 139). (C) k_{off} values from individual T cell clones bearing the identical TRAV and TRBV clonotype (n = 35). The height of the gray bars represents mean values, and each symbol represents an individual clone with EAA clones (green), ELA clones (blue), LAU 444/ELA clones (red) with a pre-existing dominant TRBV6c2 clonotype, EM28± clones (○), and EM28± clones (●).

Discussion

TCR affinity/avidity is an important determinant of thymic T cell selection (10) and T cell function (2). Reports have shown that stronger TCR:pMHC interactions generate superior effector functions in mice (19, 33–35) and humans (36–38). However, the impact of TCR/CD8:pMHC avidity on CD8 T cell differentiation and clonotype repertoire selection within the periphery remains poorly understood. In this report, we took advantage of a large library of Melan-A–specific CD8 T cell clones (n = 139) derived from melanoma patients who received vaccination with either low (native/EAA)- or high (analog/ELA)-affinity Melan-A_{MART-1} 26–35 decapeptide in combination with CpG and IFA (22). This clonal library, composed of EM28± and EM28 α clones (defined as CD8±/CD45RA±/CCR7±/CD28± or CD28 ±, respectively), served as a unique tool to study the influence of TCR:pMHC kinetics on T cell differentiation and T cell function in a well-defined, clinically relevant setting (Figs. 1, 2).

Until recently, the precise quantification of TCR:pMHC kinetics at the surface of live T cells was technically challenging. Different techniques have been developed for TCR:pMHC kinetic determination based on micropipetting and thermal fluctuation assays (39, 40). To our knowledge, the use of the novel NTAmber technology (15, 18) allowed, for the first time, the real-time quantification of dissociation along a broad dynamic range of TCR avidities (>3 logs), on live human CD8± T cells and with monomeric resolution (Fig. 3). Moreover, recently published data from our group demonstrate that TCR:pMHC dissociation (k_{off}) values derived using the NTAmber technology strongly correlate with k_{off} values obtained by SPR (20). Nauerth et al. (19) have elegantly described a Streptamer-based approach for the monomeric TCR: pMHC dissociation quantification using viral Ag. It must be noted that TCRs specific for viral Ags have a higher-affinity range than tumor-associated Ags, such as Melan-A (4). Due to their high stability and rapid reversibility (<5 s) upon addition of imidazole,
NTAmers offer increased sensitivity for low-affinity TCRs (for instance with $t_{1/2}$, $60$ s), which represent the large majority of naturally occurring TCRs specific for nonmutated tumor-associated Ags. Therefore, we chose the NTAmer technology as it eliminates the measurement biases conferred by the multivalent binding of multimers to the TCR (15, 41), allows a broad dynamic range of dissociation rates in the low-affinity spectrum, and provides high-throughput capacity with an easy-to-use experimental procedure not necessitating the generation of soluble TCRs.

Together with our unique CD8 T cell tumor Ag-specific clonal library, the NTAmer technology allowed us to accurately evaluate the impact of immunotherapy on TCR/CD8:pMHC avidity with regard to clonotype selection, differentiation, and T cell functions. By segregating the clones according to patient cohort (vaccination with native/EAA versus analog/ELA peptide) and T cell differentiation (EM28+ versus EM28-), we observed that the low-affinity (native) peptide vaccine promoted strong effector differentiation readily within the early differentiated EM28+ subset, thereby highlighting the impact of the peptide:MHC avidity during CD8 T cell priming and activation (23, 24). Moreover, our data further corroborate our previous results demonstrating that a large proportion of CD8 T cell clones derived from high-affinity (analog) vaccinated patients was unable to lyse T2-pulsed target cells (28, 42) and melanoma cell lines (22). To our knowledge, for the first time, we were able to quantify that vaccination with the low-affinity (native) peptide was associated with the presence of Ag-specific T cells bearing predominantly TCRs with high TCR/CD8:pMHC avidity (Fig. 4). Of note, although the TCR/CD8:pMHC avidity range was patient dependent, some clones derived from high-affinity (analog) peptide-vaccinated patients (LAU672 and LAU936; Fig. 4B) displayed TCR:pMHC dissociation kinetics associated with the high TCR/CD8:pMHC avidity of EAA clones. Our data support the notion that the high-affinity (analog) peptide vaccination can induce particular clones bearing high TCR/CD8:pMHC and functional avidity. However, our observations demonstrate that the low-affinity (native) peptide vaccination favors an enrichment/selection of Ag-specific clones bearing both high TCR/CD8:pMHC avidity and high functional competence. This was particularly evident in the differentiated EM28+ subset, thereby suggesting that repeated prime-boost vaccination with low-affinity peptide promoted the enrichment of high-avidity CD8 T cells during effector T cell differentiation.

Lower TCR affinities (43–45) and reduced functional avidities are typically seen in primary T cell–based immune responses compared with secondary responses. Moreover, the primary response contains a heterogeneous pool of TCR affinities, which further matures (46). Baumgartner and Malherbe (47) have found that vaccination with peptides forming pMHC complexes of low stability yielded high functional avidity CD4+ T cell responses, whereas pMHC of higher stability recruited lower-avidity clonotypes.

**FIGURE 5.** Relationship between TCR/CD8:pMHC avidity and Ca$^{2+}$ flux in vaccine-induced tumor-specific CD8 T cell clones. (A) Correlations between Ca$^{2+}$ flux (y-axis) peak mean fluorescence intensity (MFI) (left panel) and time to peak (right panel) in relationship with dissociation ($k_{off}$) rates for all vaccine-induced tumor-specific T cell clones ($n = 73$) following low-affinity (native) Melan-A$^{MART-1}_{26-35}$ peptide stimulation. Clones characterized as Ca$^{2+}$ flux deficient are represented in the gray box above the graph. (B) Correlations between Ca$^{2+}$ flux peak MFI (y-axis) in relationship with dissociation ($k_{off}$) rates based on clonal differentiation (EM28+ versus EM28-) (left and right panels) and peptide used during vaccination (native versus analog) (top and bottom panels). Each symbol represents an individual clone with EAA clones (white), ELA clones (black), pooled EAA and ELA clones (gray), EM28+ clones (○), and EM28- clones (○). Black line serves as an indicative linear regression line. Spearman’s correlation (R and p values). The $n$ values represent number of clones that displayed Ca$^{2+}$ flux capacity.
Our data are in line with previously documented observations (33, 34, 48–50) that repeated vaccination with low concentration of a low-affinity peptide (e.g., native/EAA) promotes TCR/CD8:pMHC avidity maturation, which was absent with the high-affinity (analog, ELA) cohort. We also observed that the dose of peptide could influence the quality of effector cells in vaccinated melanoma patients. Indeed, low peptide dose vaccination resulted in enhanced cytotoxicity and reduced CD8 dependence of Melan-A–specific CD8 T cells (51). Furthermore, a recent report in the RIP-OVA model suggests that high-affinity peptides could prolong the contact between T cell and APC, which favors asymmetric cell division of the daughter T cell proximal to the APC differentiating into an effector T cell (52). Conversely, CD8 T cell stimulation with low-affinity peptides results in symmetric division and reduces effector T cell differentiation. Moreover, Cole et al. (38) reported that tumor-specific T cell clones against Melan-A could have a strong preference for either the analog/ELA or the native/EAA peptide, impacting T cell function in response to either analog/ELA or native/EAA peptide stimulation. They further suggest that priming with a heteroclitic high-affinity peptide may generate tumor-specific CD8 T cells that would recognize the native Melan-A peptide suboptimally. These data together with ours (22) indicate that vaccination trials using heteroclitic analog peptides require careful re-evaluation with regard to the risk of priming T cells with imprecise Ag specificity. A clinical trial is currently under way (http://www.clinicaltrials.gov, NCT01308294) in which melanoma patients are primed with a low-affinity (native) peptide vaccine to induce high-quality Ag-specific CD8 T cells and then boosted with the high-affinity (analog) peptide to favor a maximal response (data not shown).

Finally, a major finding of this report was the demonstration of a robust correlation between TCR avidity and T cell responsiveness, in our case functional assays with Ca\textsuperscript{2+} flux (Fig. 5) and target cell killing (Fig. 6), using a large panel of tumor-specific CD8 T cell clones bearing naturally occurring TCRs. This observation has been reported by groups using either transgenically modified TCRs (37, 53–56) or pMHC variants or altered ligands (46, 48, 57, 58). In a viral setting using a microscopy and reversible Strep-tamer-based assay, slower \( k_{\text{off}} \) rates were associated with improved function and better in vivo protection (19). With a relatively large number of clones (\( n = 92 \)) expressing naturally occurring TCRs and displaying a large spectrum of TCR/CD8:pMHC avidity and functionality values, we observed that tumor-specific vaccine-induced

![FIGURE 6. Relationship between TCR/CD8:pMHC avidity and target cell killing in vaccine-induced tumor-specific CD8 T cell clones. (A) Correlations between EC\textsubscript{50} values from cytolytic assays (y-axis) in relationship with dissociation rates (\( k_{\text{off}} \)) for all vaccine-induced tumor-specific CD8 T cell clones (\( n = 92 \)) following either low-affinity (native, left) or high-affinity (analog, right) Melan-A\textsuperscript{26–35} peptide stimulation. Nonkiller clones are represented in the gray box above the graph. (B) Correlations between EC\textsubscript{50} values from cytolytic assays (y-axis) in relationship with dissociation (\( k_{\text{diss}} \)) rates based on clonal differentiation (EM28\textsuperscript{+} versus EM28\textsuperscript{−}) (left and right panels) and peptide used during vaccination (native versus analog) (top and bottom panels). Shaded gray area highlights a range of high functional avidity (EC\textsubscript{50} values between 10\textsuperscript{−10} and 10\textsuperscript{−11} M). Each symbol represents an individual clone with EAA clones (white), ELA clones (gray), pooled clones (gray), EM28\textsuperscript{+} clones (○), and EM28\textsuperscript{−} clones (□). Black line serves as an indicative linear regression line. Spearman’s correlation (R and \( p \) values). The \( n \) values represent number of clones that displayed killing capacity.](http://www.jimmunol.org/)(C) Correlations between EC\textsubscript{50} values from cytolytic assays (y-axis) in relationship with dissociation rates based on clonal differentiation (EM28\textsuperscript{+} versus EM28\textsuperscript{−})(left and right panels) and peptide used during vaccination (native versus analog) (top and bottom panels). Shaded gray area highlights a range of high functional avidity (EC\textsubscript{50} values between 10\textsuperscript{−10} and 10\textsuperscript{−11} M). Each symbol represents an individual clone with EAA clones (white), ELA clones (gray), pooled clones (gray), EM28\textsuperscript{+} clones (○), and EM28\textsuperscript{−} clones (□). Black line serves as an indicative linear regression line. Spearman’s correlation (R and \( p \) values). The \( n \) values represent number of clones that displayed killing capacity.)
T cell clones with high TCR/CD8:pMHC avidities were also the best killers. A correlation, albeit weaker, was also observed between $k_{\text{off}}$ rates and Ca$^{2+}$ flux. Our data are therefore in accordance with the dissociation rate model in which slower $k_{\text{off}}$ rates yield better functionality, and that different stimulation thresholds can trigger different functional outputs (e.g., signaling, cytokines, killing) (3).

Altogether, our work offers clear evidence that TCR/CD8:pMHC avidity is a major determinant of T cell function. The use of the novel NTAmer technology permits real-time quantification of TCR dissociation kinetics directly with monomeric resolution at the surface of primary CD8 T cells in a convenient and accessible flow cytometry–based assay. Within the scope of cancer immunotherapy based on the adoptive cell transfer of tumor Ag–specific CD8 T cells, the quantification of T cell functions in standardized assays could easily be complemented with TCR kinetics assessment with the aim to determine which cells would offer the best clinical efficiency in patients. Furthermore, this assay may identify naturally occurring TCRs with high TCR/CD8:pMHC avidity, which could be used for T cell engineering.

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Disclosures

The authors have no financial conflicts of interest.

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