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Evidence for a TCR Affinity Threshold Delimiting Maximal CD8 T Cell Function

Daphné A. Schmid,*¹ Melita B. Irving,*¹ Vilmos Posevitz,† Michael Hebeisen,‡ Anita Posevitz-Fejfar,§ J-C. Floyd Sarria,¶ Raquel Gomez-Eerland,‖ Margot Thome,¶ Tony M. Schumacher,¶ Pedro Romero,† Daniel E. Speiser,¶ Vincent Zoete,‖ Olivier Michielin,*‡,† and Nathalie Rufer*¹,†,§

Protective adaptive immune responses rely on TCR-mediated recognition of Ag-derived peptides presented by self-MHC molecules. However, self-Ag (tumor)-specific TCRs are often of too low affinity to achieve best functionality. To precisely assess the relationship between TCR–peptide–MHC binding parameters and T cell function, we tested a panel of sequence-optimized HLA-A*0201/NY-ESO1-157-165-specific TCR variants with affinities lying within physiological boundaries to preserve antigenic specificity and avoid cross-reactivity, as well as two outliers (i.e., a very high- and a low-affinity TCR). Primary human CD8 T cells transduced with these TCRs demonstrated robust correlations between binding measurements of TCR affinity and avidity and the biological response of the T cells, such as TCR cell-surface clustering, intracellular signaling, proliferation, and target cell lysis. Strikingly, above a defined TCR–peptide–MHC affinity threshold (Kₐ < ~5 nM), T cell function could not be further enhanced, revealing a plateau of maximal T cell function, compatible with the notion that multiple TCRs with slightly different affinities participate equally (codominantly) in immune responses. We propose that rational design of improved self-specific TCRs may not need to be optimized beyond a given affinity threshold to achieve both optimal T cell function and avoidance of the unpredictable risk of cross-reactivity.

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Similar to the narrow window of TCR-Ag binding affinities resulting from thymic selection and self-tolerance mechanisms (7), various models have predicted that T cell activation may be limited to a window of affinities for the TCR-pMHC interaction and that above or below this window, T cells may not develop productive functions (8). In that context, selective loss of Ag-specific clonotypes that expressed low TCR-pMHC affinity has been observed, indicating that a minimum threshold of TCR binding must be achieved to clonally activate and select specific Th cells (17). Studies based on cytokine secretion or killing assays using peptide-loaded APCs as targets have shown that the minimum concentration of Ag required to elicit a T cell response in terms of functional avidity was \( \approx 1 \mu M \) (18, 19). In contrast to these findings, several reports have recently examined T cells bearing engineered TCRs variants with avidities in the nanomolar range (20, 21) and found that reports have recently examined T cells bearing engineered TCRs variants with affinities in the nanomolar range (20, 21) and found enhanced T cell function (22–26). However, when TCR affinity is increased to high and supraphysiological avidities, T cells react with many different pMHC complexes and may lose defined Ag specificity, leading to dangerous cross-reactivity (24, 25, 27).

An important aspect often neglected by these studies is a detailed assessment of the impact of each optimized TCR variant on the TCR-pMHC binding avidity, downstream signaling, and functional avidity in engineered T lymphocytes. To specifically address this issue, we assessed a panel of affinity-optimized TCR variants specific for the tumor Ag A*0201/NY-ESO-1\(^{157-165}\) for pMHC binding and T cell function. These TCRs have been generated by a novel structure-based approach (28, 29), allowing the rational design of TCRs that preserve precise antigenic specificity and avoid cross-reactivity, unlike previously designed TCRs (20, 21). In the current study, we observed slower TCR-pMHC binding off-rates, increased TCR-pMHC multimerization, and intracellular signaling through p-linker for activation of T cells (LAT) and p-ERK1/2 in T cells expressing high-affinity TCR variants, supporting enhanced T cell functionality. Importantly, above a defined TCR affinity threshold, T cell avidity and function were not further enhanced, thus delimiting a plateau for maximal activity. Altogether, our data indicate that TCRs may not be needed to be optimized beyond a given affinity threshold to achieve best functionality.

Materials and Methods

**Cell lines and primary CD8 T lymphocytes**

SUP-T1, melanoma cell lines (Me 275, Me 290, and NA8), T2-A2 (TAP-deficient lymphoblastoid cell line transfected with HLA-A*0201), C1R-WT, and C1R-CDS\(^{\text{mutt}}\) cells were cultured in RPMI 1640 (Invitrogen, Basel, Switzerland) supplemented with 10% FCS, 10 mM HEPES, penicillin (100 U/ml), and streptomycin (100 \( \mu \)g/ml). PBMCs were obtained from two healthy donors by density centrifugation using Ficol-Hypaque (Pharmacia Biotech, Uppsala, Sweden). CD8 T lymphocytes were positively enriched using anti-CD8-coated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and were either cryopreserved for later use or expanded posttransduction in RPMI 1640 medium supplemented with 8% human serum, 150 U/ml recombinant human IL-2 (3000 (GE Healthcare, Glattbrugg, Switzerland).

**Generation of lentiviral constructs encoding WT and variant TCRs**

The full length codon-optimized TCR AV23.1 and TCR BV13.1 [according to Arden’s nomenclature (30)] chain sequences of a dominant T cell clone (TCC) of patient LAU 155 (31) were cloned in the pRRL lentiviral (LV) transfer vector, a third-generation HIV-based LV vector, kindly provided by L. Naldini (32), in which most of the U3 region of the 3’ long terminal repeat was deleted, resulting in a self-inactivating 5’ long terminal repeat, or SIN. The most efficient LV construct consisted of the codon-optimized TCRs-chain under the hPGK promoter, linked to the codon-optimized TCRβ-chain by an internal ribosomal entry site (IRES) and a kozak sequence to enhance translational efficiency (data not shown). Structure-based amino acid TCR substitutions were introduced into the WT TCR sequence using the QuickChange MultiSite-Directed Mutagenesis Kit (Stratagene) and confirmed by DNA sequencing.

**LV production and cell transduction**

LV vectors were produced by transient transfection of 293T cells using a standard calcium phosphate precipitation protocol. In brief, 293T cells were cotransfected with the vector of interest (pRRL-hPGK-TCR AV23.1-IRES-TCR BV13.1 and the LV transfer vector, envelope, and packaging plasmids (pRSV-Rev, pMD2-VSV-G, and pMDLg/pRRE). LV supernatants were harvested 24 h and 48 h posttransfection, filtered, and concentrated by ultracentrifugation. Pellets were resuspended in the appropriate volume of sterile cold PBS and either stored at –80°C or directly used. A total of 1 \times 10^6/ml SUP-T1 cells or 2 \times 10^6/ml CD8 T cells were transferred to pretreated polybrene plates (1 \mu g/ml and transduced with concentrated LV supernatant. Expression of transduced TCRs was measured by flow cytometry on d5 posttransduction. LV production and cell transduction were conducted in at least three independent experiments and produced comparable results. Noting no major biases in the expression of the introduced TCRs.

**Flow cytometry analysis**

SUP-T1 and CD8 T cells expressing WT or variant NY-ESO-1-specific TCRs were stained with PE-labeled HLA-A2/NY-ESO-1\(^{157-165}\) (SLLMWITQA) multimers as described above (33) and/or with PE- or FITC-conjugated Abs against V13.1 (Beckman Coulter, Nyon, Switzerland), CD4, or CD8 (Beckton Dickinson, San Diego, CA). Cross-reactivity of TCR variants was assessed as described above using PE-labeled A2/CMY (pp65, NLV-PMVAT), A2/FluMA (GILGFVFTL), A2/Melan A\(^{26-35}\) (ELAGILGVT), A1/MAGE3 (EVDPIGHLY), and A3/FluMA (RLEDVFAGK) multimers. Flow cytometry analyses were performed on an LSR II flow cytometer (BD Biosciences, San Jose, CA), and data were analyzed using CellQuest (Tampa, FL), FCS Express (De Novo Software, Los Angeles, CA) or FlowJo software (Tree Star, Ashland, OR). Bulk CD8 T cells transduced with WT and variant TCRs were further enriched following sorting of multiple individual T cells using an FACSVantage SE machine (BD Biosciences) and included CD8 T cells expressing the V491 TCR variant, despite the reduced proportion of multimer“-stained cells. To allow direct comparison between the different transduced CD8 T cells, we used in every experiment cell cultures that expressed similar levels of the TCR BV13 (ranging between 90% and 99%).

**Multimer association and dissociation measurements**

For association (on-rates) experiments, SUP-T1 expressing WT or variant NY-ESO-1-specific TCRs were first stained for 20 min at 4°C with FITC-labeled anti-CD4 mAb, washed once, and resuspended in buffer. An aliquot (corresponding to time \( t_0 \)) was determined by flowcytometry. PE-labeled A2/NY-ESO-1\(^{157-165}\) multimers (2 \( \mu \)g/ml) were then added to the samples, aliquots were collected at different time points, and intensity of stainings was measured by flow cytometry. Transduced CD8 T cells were first stained with PE-labeled anti-multimers. For dissociation experiments (off-rates), SUP-T1 and CD8 T cells transduced with WT or variant TCRs were first stained with PE-labeled A2/NY-ESO-1\(^{157-165}\) multimers, followed by SUP-T1 cells by staining with FITC-labeled anti-CD4 mAb as described above. An aliquot (corresponding to time \( t_0 \)) was taken and directly analyzed by flow cytometry. A total of 5 \mu g/ml OKT3 was added to the remaining samples to avoid rebinding of the multimers after dissociation experiments. Maximal binding and \( k_{on} \) (min\(^{-1}\)) were determined.
Confluent TCR-transduced SUP-T1 cells were washed once in RPMI 1640 medium and pellets resuspended at 3 × 10^6 density/ml. Staining was performed either with PE-labeled A2/NY-ESO-1_{157-165} or A2/CMV_{495-503} multimers at 4.4 μg/ml. A total of 30 μl cell suspension was pipetted per spot onto a 12-spot slide (Marienfeld Laboratory Glassware, Landau-Königshofen, Germany) and incubated for 1 min at 37°C. To achieve proper attachment of the cells to the surface, the slides were placed for a further 10 min to 4°C and remaining nonattached cells were washed away in PBS. The cells were fixed in 4% paraformaldehyde. Following three washing steps, nuclei were stained with Hoechst 33342. Then, after a manual selection around cell membrane (interactive pen display, Cintiq 21UX, Wacom, Saitama, Japan), a second journal measured the surface of the cell and cut its border by an erosion of 2 μm, allowing the quantification of surface (high-intensity clusters) and total fluorescence of the cell membrane fluorescence.

Western blot analysis

Typically, 2 × 10^6 CD8 T cells per lane or 1 × 10^6 SUP-T1 cells per lane were used for biochemical analysis. For all experiments, transduced CD8 T cells or SUP-T1 cells were either left unstimulated or stimulated with OKT3 (10 μg/ml) for 10 min or with A2/NY-ESO-1_{157-165} multimer (10 μg/ml) at indicated time points at 37°C in RPMI 1640. All stimulations were performed in the presence of 10 μg/ml anti-CD28 Ab (BD Biosciences). Cell extracts were obtained by resuspending the pellets in lysis buffer containing 1% Nonidet P-40, 1% lauryl maltoside (ν-dodecyl-β-D-maltoside), 50 mM Tris (pH 7.5), 140 mM NaCl, 10 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 mM NaVO_4. Postnuclear supernatants were subjected to immunoblotting. Proteins were separated by SDS-PAGE followed by electrotransfer to nitrocellulose membranes. Subsequently, membranes were probed with the following Abs: anti–phospho-tyrosine 4G10 (Upstate/Millipore, BioConcept, Allschwil, Switzerland), anti–phospho-ERK1/2 (Cell Signaling Technology, BioConcept, Allschwil, Switzerland), anti–phospho-LAT (LAT Y-171, and anti–actin mouse Ab (clone AC-15, Sigma-Aldrich). Blots were washed and reprobed with 1% Nonidet P-40, 1% lauryl maltoside, 50 mM Tris (pH 6.7) at 56˚C for 25 min, then washed and reprobed with anti–β-actin mouse Ab (clone AC-15, Sigma-Aldrich).

CFSE proliferation and chromium release assays

CFSE-labeled CD8 T cells (0.5 × 10^6) transduced with WT or variant TCRs were incubated with T2 target cells (0.5 × 10^5) pulsed with analog NY-ESO-1_{157-165} peptide (0.01 μg/ml, SLLMWITQA) or CMV pp65 peptide (1 μg/ml, NLVPMVQTV) in RPMI 1640 supplemented with 8% human serum. A total of 10 U/ml IL-2 was added 48 h poststimulation. On days 3 and 4, 2 × 10^4 cells were collected and analyzed on an LSR II flow cytometer (BD Biosciences). The percentage of CD8 T cells proliferating (CFSE^{win}) in response to NY-ESO-1–specific stimulation was estimated from the proportion of corresponding CFSE-labeled cells (CFSE^{win}) stimulated with the irrelevant CMV peptide.

Lytic activity and Ag recognition was assessed functionally in 4 h [^{3}Cr]-release assays using: 1) T2 target cells or C1R target cells that expressed either WT or CD8-null HLA-A2 (HLA-A2/77AP^{–}/–) pulsed with serial dilutions of analog NY-ESO-1_{157-165} peptide [SLLMWITQA (34)], or Melan-A_{26-35} A2T, peptide (ELAGIGILTV); and 2) the melanoma cell lines Me 275 (A2^{–}/NY-ESO-1^{–}), Me 290 (A2^{–}/NY-ESO-1^{–}), and NA8 (A2^{–}/NY-ESO-1^{–}) incubated in the presence or absence of the analog NY-ESO-1_{157-165} Peptide. The percentage of specific lysis was calculated as 100 × (experimental – spontaneous release)/total – spontaneous release.

Statistics

The results were analyzed by unpaired two-sample t test, one-phase exponential decay, log–log linear regression analyses, and log sigmoid curve fitting using GraphPad Prism version 5.02 (GraphPad, San Diego, CA).

Results

Selection of a panel of re-engineered NY-ESO-1–specific TCR variants with progressive increased affinities

Recently, we identified dominant T cell clonotypes from melanoma patient LAU 155 who mounted a strong natural immune response against the cancer testis antigenic epitope HLA-A*0201/NY-ESO-1_{157-165} (31, 35). One of them expressed the TCR AV23-BV13 (named BV13-clonol1) that is closely related to the IG4 TCR for which a crystal structure (2BNR in the Protein DataBank) has been reported (36). The sequence of IG4 differs from BV13-clonol1 by only 4 aa residues, two within the CDR3α loop (T95Q and S96T) and two within the CDR3β loop (N97A and T98A). This experimental structure (2BNR) allowed the application of a novel in silico structure-based TCR approach to rationally design sequence mutations of the BV13-clonol1 TCR (28, 29 and V. Zoete, M. Irving, M. Ferber, D. Schmid, N. Rufer, and O. Michielin, manuscript in preparation). Based on these in silico calculations, five NY-ESO-1–specific TCR variants were selected for this study, with amino acid replacements in CDR2 (V49, G50, A51) and CDR3 loops of the TCR BV13-clono1 (LAU 155) were modified by guest on April 16, 2017 http://www.jimmunol.org/ Downloaded from

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<td>V49I</td>
<td>S I G A G I G A A G n.a n.a</td>
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<tr>
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<tr>
<td>G50A+A51E</td>
<td>S V A E G I G A A G 1.9 2.4 × 10^{–2} 0.20</td>
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<td>A97L</td>
<td>S V A A G I G A A G 2.7 2.3 × 10^{–2} 0.05</td>
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<tr>
<td>1G4 (36)</td>
<td>S V G A G I G A A G 14.2 1.2 × 10^{–2} 0.17</td>
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<tr>
<td>wtc51 (modified)</td>
<td>S V A I Q T G A A G 0.015 8.5 × 10^{−6} 0.0013</td>
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TCR variants, with amino acid replacements within CDR2 loops of the TCR BV13-clono1 (LAU 155) were selected for this study, with amino acid replacements in CDR2 (V49, G50, A51) and CDR3β (A97) (Table I). Residues V49, G50, and A51 mostly interact with the HLA-A*0201 molecule, whereas A97 primarily binds to the NY-ESO-1 peptide. We characterized the affinity (Table I) of these mutants and an additional triple TCR variant that combines G50A, A51E (CDR2β),
and A97L (CDR3α) substitutions for the pMHC complex by SPR. When compared with the WT TCR (KD, 21.4 μM), TCRs variants showed an incremental hierarchy in affinity from single mutants (A51E < G50A < A97L) to the double mutant G50A+A51E (KD, 1.9 μM), and the triple mutant G50A+A51E+A97L (KD, 0.9 μM; 23-fold increase). We did not assess the V49I TCR variant, because it displayed very poor binding to pMHC when titrated by ELISA (data not shown).

FIGURE 1. TCR–pMHC binding kinetics in primary CD8 T cells transduced with WT and variant TCRs. A, TCR surface expression and specificity was evaluated in transduced bulk CD8 T cells by flow cytometry analysis using anti-BV13 Ab and A2/NY–ESO-1 multimers. Control stainings with A2/CMVpp65 are depicted. Stainings of cells transduced with TCR 1G4 and wtc51 gave similar results (data not shown). To allow direct comparison between samples, we always used CD8 T cells expressing >90% of transduced TCR BV13. Of note, the flow cytometry profiles represent a compiled set of data obtained from different experiments, accounting for some interexperimental variations in terms of MFI. The surface expression of each TCR variant was quantified in subsequent experiments described in B through Fig. 6. B, The rate of association of multimers on transduced CD8 T cells was measured as described in Materials and Methods. C, After staining transduced CD8 T cells with multimers, decay of staining was measured by flow cytometry over time. The percentage of initial bound multimer that remained associated with the cells after various time points is depicted as a representative experiment, and time for half maximal binding (t1/2) was determined. D, Average koff values (dissociation constant of the pMHC from the TCR, min⁻¹) assessed during multimer dissociation assays and representative of eight independent experiments. Data were analyzed by unpaired two-sample t test. The dotted line was arbitrarily set at the koff value obtained for WT TCR and allows direct comparison between the different transduced CD8 T cells. We also included the natural TCC from which the WT TCR BV13-clonol had been originally isolated. E, Relationships between multimer off-rates (koff, min⁻¹) and monomeric affinity (KD, μM) or off-rates (koff, s⁻¹) as measured by SPR expressed as log–log fitting straight lines.
Cell-surface binding avidity of NY–ESO-1–specific TCR variants strongly correlating with their respective soluble TCR–pMHC affinity

All TCR variants specific for NY–ESO-1/A2 were transduced in SUP-T1 cells (37) and primary bulk CD8+ T cells using the VSV-G pseudotype third generation of LV vectors. We also included the closely related 1G4 TCR (36) and an adapted wtc51 TCR previously selected upon bacteriophage library screening for its nanomolar range of affinity toward the A2/NY–ESO-1 complex (21) (Table I). Together with WT (BV13-clono1) and 1G4 TCRs, these TCR variants represent a selection of NY–ESO-1–specific TCRs that can be classified according to an incremental hierarchy of monomeric-soluble TCR–pMHC affinities (Table I). We sought to determine whether a progressive increase in multivalent TCR–pMHC binding avidities would similarly be observed when assessed at the surface level of T lymphocytes. To address this issue, we first measured the percentage of specific multimer binding by SUP-T1 (Supplemental Fig. IA) and bulk CD8 T (Fig. 1A) cells transduced with the different TCR variants. All transduced cells expressed comparable levels of TCR Vβ13 as assessed by staining with an anti-BV13 mAb. With the exception of the V49I TCR mutant, the proportion of multimer-positive T cells was comparable, ranging between 88% and 99%. This result indicates that V49I TCR mutant likely forms TCR–pMHC complexes of relative low stability at the surface of T lymphocytes, in accordance with the finding that the soluble version of this TCR poorly binds the pMHC (data not shown). Moreover, we also observed that none of the transduced T cells had significant binding for: 1) pMHC presenting irrelevant peptide epitopes (i.e., CMV, flu, Melan-A); or 2) allelic variants of HLA-A1 or A3 multimers (Fig. 1A; data not shown).

We then assessed cell surface-bound multimer on-rates (Fig. IB, Supplemental Fig. 1B) and off-rates (Fig. 1C; Supplemental Fig. 1C). Analysis of multimer association rates revealed a rapid initial increase of the MFI for all transduced cells, either within the first hour (SUP-T1 cells) or first 20 min (CD8 T cells), accompanied by substantial MFI differences between the TCR variants after 120 min. Indeed, V49I TCR mutant always showed the lowest mean fluorescence at equilibrium, whereas G50A, A97L, G50A+A51E, and G50A+A51E+A97L mutants systematically had higher MFI than the WT TCR. Importantly, the dissociation kinetics varied between the TCR variants and could be classified into the following hierarchy: V49I < WT = 1G4 < A51E < A97L < G50A = G50A+A51E < G50A+A51E+A97L < wtc51 (Fig. 1C, Supplemental Fig. 1C). Comparable rates of association and dissociation were observed between WT (BV13-clono1) and 1G4 TCRs that represent the two natural unmodified TCRs. Remarkably, the dissociation rate constant (koff, s−1) of the pMHC from the TCR variants in transduced T cells (Fig. 1D, Supplemental Fig. 1D) showed excellent correlation with the monomeric TCR–pMHC affinities (Kd) as well as with dissociation rates (koff, s−1) measured by SPR (Fig. 1E, Supplemental Fig. 1E). Collectively, these results support the notion that TCR multimer off-rates directly and strongly correlate with monomeric TCR–pMHC affinities, being faster for TCRs of relatively low affinity (e.g., V49I, WT, 1G4, and A51E) and slower for TCRs of higher affinities (e.g., A97L, G50A, G50A+A51E, and G50A+A51E+A97L). In line with these results, both SUP-T1 cells and CD8 T cells expressing the modified wtc51 TCR variant of nanomolar range affinity exhibited the slowest multimer off-rates (with an average mean t1/2 of 62 min for CD8 T cells). Of note, the natural TCC from which the WT TCR BV13-clono1 had been originally isolated (31) depicted slower off-rates when compared with CD8 T cells transduced with the same WT TCR (Fig. 1). This effect may best be explained by the higher level of expressed TCRs (increased MFI) by the original clone that could impact both the association and dissociation rates through increased pMHC binding avidity (8).

Enhanced multivalent clustering of a TCR variant with increased affinity in cell membranes of SUP-T1 cells

We next examined fluorescence intensity and clustering of TCR–pMHC complexes in transduced SUP-T1 cells at the single-cell level using scanning confocal microscopy, deconvolution, and image processing (Fig. 2). In accordance with the TCR–pMHC affinity and binding avidity data, SUP-T1 cells transduced with the G50A+A51E TCR variant exhibited increased clustering of TCR–pMHC complexes in individual cells when compared with SUP-T1 cells expressing WT or V49I TCRs. Moreover, we observed statistically significantly stronger fluorescence intensity signals in the cytoplasm, suggesting enhanced internalization of the TCR–pMHC complex in those cells (Fig. 2). In contrast, both relative intensity fluorescence and frequency of high intensity clusters per cell were strikingly reduced for the V49I TCR mutant, in line with the observation that this particular TCR possesses poor binding avidity for the A2/NY–ESO-1 complex.

Higher levels of LAT and ERK phosphorylation and increased proliferation in CD8 T cells transduced with a high-affinity TCR variant

Several studies have shown that multivalent clustering of TCRs is necessary for T cell signaling and activation (38, 39). Therefore, we examined whether the increase in TCR–pMHC clustering

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**FIGURE 2.** Immunofluorescence stainings of SUP-T1 cells expressing WT TCR or V49I or G50A+A51E TCR variants. A. Cells were stained with PE-conjugated HLA-A2/NY–ESO-1 multimers prefixation and Hoechst 33342, and analyzed by confocal microscopy (original magnification ×63). Individual cell images represent an additional 4-fold of magnification. B. After acquisition, the raw images were deconvolved, processed, and quantified as detailed in Materials and Methods. All data are representative of three independent experiments. Single cells expressing WT or V49I or G50A+A51E TCRs are depicted.
A2/NY-ESO-1+CD28

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OKT3+CD28

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CD8+

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FIGURE 3. Levels of LAT and ERK phosphorylation in SUP-T1 and CD8 T cells expressing TCR variants. A, TCR-untransduced (○) or TCR-transduced (WT, G50A+A51E, V49I) SUP-T1 or CD8 T cells were stimulated for 10 min at 37°C with A2/NY-ESO-1157-165 multimer or with OKT3 in the presence of anti-CD28 mAb. All data are representative of at least three independent experiments. B, CD8 T cells transduced with WT TCR or G50A+A51E variant were left unstimulated (t = 0) or were stimulated at 37°C for 1, 5, 10, 15, and 20 min with A2/NY-ESO-1157-165 multimers in the presence of anti-CD28 mAb. Data are representative of four independent experiments. C, To allow direct comparison between the different time-points, intensity of LAT and ERK2 phosphorylation levels relative to unstimulated samples (t = 0, arbitrarily set as 1) were quantified and subsequently normalized to β-actin. Inserts show the phosphorylation baseline of unstimulated cells (WT versus G50A+A51E) as assessed by antiphosphotyrosine stainings. Mean values ± SEM are shown in each graph. A–C, Cell lysates were assayed for levels of LAT (p-Tyr and Y171) and ERK/1/2 phosphorylation. β-actin was used as a loading control to compare protein levels between samples. All transduced T cells expressed comparable proportions of transduced TCR BV13. D, Relative proliferative capacity of CD8 T cells transduced with TCR variants normalized to the proliferative capacity of WT transduced T cells (represented by the dotted line). CFSE-labeled transduced CD8 T cells were assessed by flow cytometry at days 3 or 4 poststimulation with T2 cells pulsed with 0.01 μg/ml NY-ESO-1157-165 peptide as described in the Materials and Methods. Data from nine independent experiments are depicted.
variants, shared similar functional avidity, as they required similar peptide concentrations to achieve half-maximal lysis of T2 cells. The second group comprised mostly cells expressing TCR variants of increased binding affinity/avidity to pMHC, namely G50A, G50A+A51E, A97L, and G50A+A51E+A97L, and demonstrated statistically significant superior functional avidity over WT cells (median of 50% maximal target cell lysis, 0.04 nM versus 0.18 nM). Remarkably, the concentrations of NY–ESO-1157–165 peptide that yielded half-maximal activity were highly similar for the different members of this group and correlated to the activity observed for the natural TCC (data not shown).

Altogether, our results reveals a robust correlation between multimeric TCR–pMHC binding off-rates ($k_{\text{off}}$, min$^{-1}$) and functional killing activity; there was a drastic reduction in killing function of T cells expressing the lowest binding avidity TCR (e.g., V49I), and, conversely, enhanced function in those cells of highest TCR binding avidities (Fig. 5A). Our results also show a nice correlation between T cell functional avidity and monomeric TCR–pMHC affinity. Importantly, above a given TCR affinity threshold (delineated by the affinity of the A51E TCR variant, $K_D < 7.1 \mu$M), T cell function could not be further enhanced as demonstrated by the comparable functional avidities obtained for the second group of transduced T cells (Fig. 5A, circles). Finally, bulk CD8 T cells that stably expressed the wtc51 TCR variant of nanomolar affinity showed an unexpected reduced functional avidity when compared with WT cells (EC$_{50}$, 0.86 ± 0.3 nM versus 0.18 ± 0.1 nM, respectively; Fig. 4).

CD8 T cells expressing TCRs of increased affinity exhibit lower CD8 dependency

To analyze CD8 dependency of target cell recognition by CD8 T cells expressing TCRs of progressive affinities, C1R cells transfected with mutant HLA-A2 molecules that abrogate CD8 binding (42) were used as target cells (Fig. 5B). WT TCR and variants A51E and A97L exhibited inferior functional avidity of Ag recognition compared with cells expressing variants G50A +A51E and G50A+A51E+A97L. In contrast, T cells expressing the low avidity receptor V49I did not recognize C1R CD8 null target cells at any concentration of NY–ESO-1157–165 peptide tested. These data indicate that T cells bearing TCRs with higher binding strength ($K_D \leq 1.9 \mu$M) are less dependent for CD8–MHC interactions than ones expressing TCRs of weaker affinities.

Specific and enhanced tumor cell recognition by CD8 T cells expressing TCR variants of increased affinity

Finally, we investigated the capacity of CD8 T cells transduced with TCRs of varying affinities to specifically recognize and lyse tumors expressing the naturally processed NY–ESO-1 epitope (Fig. 6). Except for V49I, all of the transduced T lymphocytes efficiently killed the Me 275 and Me 290 melanoma tumor cell lines (Fig. 6A). The relative tumor-killing activity of transduced T cell variants was estimated as the ratio of the percentage of specific lysis obtained without adding exogenous NY–ESO-157–165 peptide (Fig. 6A) versus that obtained after adding exogenous peptide (Fig. 6B). T cells transduced with V49I variant exhibited a ratio close to 0, indicating that such cells were unable to recognize tumor cells when no exogenous peptide was added (Fig. 6C). In contrast, WT cells with ratios around 0.5 showed intermediate tumor cell recognition and lysis. Remarkably, tumor reactivity was progressively enhanced up to ratios of close to 1 for T cells expressing TCR variants of higher affinity, corresponding with their incremental affinity hierarchy. These results demonstrate that such T cell variants have the ability to strongly recognize NY–ESO-1 naturally expressing tumor cells (Fig. 6A, 6C). However, this effect became abrogated for the two TCR variants of highest affinity (G50A+A51E+A97L, wtc51), and this was particularly evident for

![Image of Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Efficiency of NY–ESO-1 Ag recognition by CD8 T cells transduced with TCR variants. A. The relative TCR avidity of each variant (filled symbols) was compared with WT transduced T cells (open squares) using T2 target cells (HLA-A2$^+$/TAP$^{-/-}$) loaded with graded concentrations of analog (SLLMWITQA) NY–ESO-1157–165 peptide. For each experiment, we included T2 cells pulsed with the irrelevant Melan-A26–35 peptide (data not shown). B. Complete collection of data ($n = 16$ independent experiments) representing the peptide concentration (in log scale) used to achieve 50% of maximal lysis. WT TCR and TCR variants are classified according to their progressive increased affinities (by SPR). Each data point represents the result of an individual transduced CD8 T cell. Statistically significant $p$ values are shown (unpaired two-sample $t$ test). Of note, similar data were obtained when transduced T cells were tested against T2 cells pulsed with the native (SLLMWITQC) NY–ESO-1157–165 peptide (data not shown).
A major finding of this study is the remarkable correlation between monomeric soluble TCR–pMHC binding affinity (K_D) and monomeric TCR–pMHC dissociation kinetics on T cells (11/2), with faster off-rates for TCRs of relatively low affinity as compared with TCRs of increased affinity (Fig. 1, Supplemental Fig. 1). This was particularly evident for the V49I- and wtc5I-modified TCR variants showing the fastest and slowest dissociation rates, respectively. Strong correlations were not only found with transduced SUPT1 cells expressing relatively low levels of the CD8 coreceptor, but also with primary CD8 T cells. Collectively, elevated TCR affinities resulted into the slower dissociation rates of pMHC multimers from downstream signaling, and subsequent T cell function.

In this study, we characterized a selected panel of TCR variants specific for the pMHC ligand A2/NG–ESO-1157–165 and derived from the original TCR BV13-clono1 (31). These TCR variants were designed by a novel structural-based modeling approach allowing the step-by-step increase of the affinity to the TCR in a highly controlled manner (28, 29) and V. Zoete, M. Irving, M. Ferber, D. Schmid, N. Rufer, and O. Michielin, manuscript in preparation). The latter relies on: 1) the identification of individual amino acid residues of defined importance for the TCR–pMHC interaction and binding; 2) structure-based design of corresponding putative sequence modifications; and 3) their selection based on the calculated binding free-energy change upon mutation. Affinities of the predicted TCRs followed an incremental hierarchy from 21.4 μM (WT) up to 0.91 μM (G50A+A51E+A97L), lying within physiological boundaries (6) and below the affinity threshold described for Ag cross-reactivity (25). We also included two outliers. The first one comprised the V49I TCR variant, the only TCR predicted to have an unfavorable ΔΔG_binding Value and for which titration ELISA revealed extremely low levels of binding to pMHC (data not shown). The second outlier assessed, the TCR wtc5I, was adapted from Dunn et al. (21) and had the highest affinity of all TCRs characterized (K_D = 15 nM).

Affinity and avidity of NY–ESO-1–specific TCR variants
clustering and multimerization process. In this study, we demonstrate that T cells transduced with the TCR variant (G50A+A51E) of increased affinity and relatively slow off-rate underwent enhanced TCR aggregation and clustering upon engagement with multimers, which may account for the markedly enhanced LAT and ERK1/2 phosphorylation and proliferation of those cells (Figs. 2, 3). In sharp contrast, the cells transduced with the V49I variant, having the lowest binding capacity for pMHC, displayed less frequent multimerization per cell, barely detectable levels of phospho-ERK, and limited proliferation and tumor killing. This is to our knowledge the first time that the impact of single or dual TCR amino acid replacement has been comprehensively assessed and directly translated into positive (e.g., G50A, A97L, and G50A+A51E), in stark contrast with T cells expressing TCR variants of faster off-rates (e.g., V49I and WT), which displayed poor or less efficient functional avidity (Fig. 5). Secondly, TCR variants with the slowest dissociation rates (e.g., G50A+A51E+A97L and wtc51-modified) showed reduced killing of cognate tumor cell lines (Fig. 6), suggesting that these prolonged $k_{off}$ constant rates may lie

**FIGURE 6.** Melanoma cell killing by CD8 T cells transduced with TCR variants. A and B, Tumor reactivity for the melanoma cell lines Me275 and Me290 (A2+/NY–ESO-1+), and NA8-MEL (A2+/NY–ESO-1−) in the absence (A) or presence (B) of analog NY–ESO-1157–165 peptide (1 μM) at the indicated E:T ratio. Dotted lines are set at 50% of specific lysis to allow direct comparison between transduced CD8 T cell variants. C, Killing ratio was estimated from the percentage of specific lysis obtained without adding exogenous peptide (A) versus the proportion of lysis obtained after adding exogenous peptide (B) at an E:T ratio of 10:1. WT TCR and TCR variants are classified according to their progressive increased affinities (by SPR). Dotted lines are arbitrarily set at the average killing ratio values obtained for WT T cells and allow direct comparison between the different CD8 T cell variants. Data are representative of 12 independent experiments. D, Model integrating the relationship between functional avidity (EC50) and monomeric TCR affinity ($K_D$) of CD8-transduced TCR WT and variants.
TCRs had an affinity above a given threshold showed enhanced T cell antitumor response. Indeed, those T cells for which the point to the existence of a TCR affinity threshold for maximal enhancing intracellular signaling and lowering the threshold of both stabilizing the binding of TCR to pMHC complexes and bridomas and found a relatively sharp affinity cutoff between full activity or no activity, which likely defines the CD8 requirement threshold. Indeed, CD8 molecules likely play an important role by both stabilizing the binding of TCR to pMHC complexes and enhancing intracellular signaling and lowering the threshold of T cell activation (reviewed in Ref. 8). Strikingly, our data also point to the existence of a TCR affinity threshold for maximal T cell antitumor response. Indeed, those T cells for which the TCRs had an affinity above a given threshold showed enhanced killing avidity when compared with WT T cells, but only up to a maximal activity plateau (equivalent to an average EC_{50} peptide concentration of 0.04 nM) (Fig. 4). Not surprisingly, with increased TCR affinities, the T cells became less CD8 dependent (Fig. 5) (42). Altogether, our data provide new evidence that the CD8 T cell function is controlled within a given window of TCR-pMHC binding affinities. On the one hand, minimal TCR affinity is needed for T cell activation, also defined as the agonist threshold, and nicely illustrated in this study by the V491 TCR variant (Fig. 6D). On the other hand, T cells are also defined by a plateau of maximal activity revealing a TCR affinity threshold (depicted in our model by K_{D} < A51E TCR variant). Overall, these data indicate that there is no need to optimize TCRs for pMHC binding above a certain affinity threshold, because this may not lead to further enhanced activity.

The native WT TCR BV13-clono1 isolated from patient LAU 155 already shows very good affinity/avidity and induces a functional activity closely related to the plateau of optimal T cell reactivity to MAGE-A10(254-262): high avidity-specific cytolytic T lymphocytes 

outside the optimal range for efficient T cell functionality (i.e., serial triggering may be limiting). Indeed, this rather surprising observation was particularly evident for the wtC1 TCR variant (K_{D} of 15 nM), which displayed both limited functional avidity (as measured by EC_{50}) and tumor-killing activity. Increased TCR affinity, up to the nanomolar range, has mostly been associated with the loss of target cell specificity (24, 25). Our data now indicate that increases in T cell reactivity may also be accompanied by a significant reduction in the specific antitumor T cell response (Fig. 6D).

Recently, Chervin and coworkers (53) investigated the specific role of the CD8 coreceptor on a large panel of 2C TCR affinity variants specific for SIYR/K\(^\beta\). In their study, they measured the IL-2 release by transduced CD8 coreceptor-negative T cell hybrids and found a relatively sharp affinity cutoff between full activity or no activity, which likely defines the CD8 requirement threshold. Indeed, CD8 molecules likely play an important role by both stabilizing the binding of TCR to pMHC complexes and enhancing intracellular signaling and lowering the threshold of T cell activation (reviewed in Ref. 8). Strikingly, our data also point to the existence of a TCR affinity threshold for maximal T cell antitumor response. Indeed, those T cells for which the TCRs had an affinity above a given threshold showed enhanced killing avidity when compared with WT T cells, but only up to a maximal activity plateau (equivalent to an average EC_{50} peptide concentration of 0.04 nM) (Fig. 4). Not surprisingly, with increased TCR affinities, the T cells became less CD8 dependent (Fig. 5) (42). Altogether, our data provide new evidence that the CD8 T cell function is controlled within a given window of TCR-pMHC binding affinities. On the one hand, minimal TCR affinity is needed for T cell activation, also defined as the agonist threshold, and nicely illustrated in this study by the V491 TCR variant (Fig. 6D). On the other hand, T cells are also defined by a plateau of maximal activity revealing a TCR affinity threshold (depicted in our model by K_{D} < A51E TCR variant). Overall, these data indicate that there is no need to optimize TCRs for pMHC binding above a certain affinity threshold, because this may not lead to further enhanced activity.

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