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

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

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-ESO-165–165–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 cell clones 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 μM), 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. The Journal of Immunology, 2010, 184: 4936–4946.

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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 ≈1 µM (18, 19). In contrast to these findings, several reports have recently examined T cells bearing engineered TCRs with affinities in the nanomolar range (20, 21) and found enhanced T cell function (22–26). However, when TCR affinity is enhanced to very high and supraphysiological affinities, 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 Aβ’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 need 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 MLA-A’0201), C1R- and C1R-CDSnull cells were cultured in RPMI 1640 (Invitrogen, Basel, Switzerland) supplemented with 10% FCS, 10 mM HEPES, penicillin (100 U/ml), and streptomycin (100 µg/ml). PBMCs were obtained from two healthy donors by density centrifugation using Ficoll-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 future use or expanded posttransduction in RPMI 1640 medium supplemented with 8% human serum, 150 U/ml recombinant human IL-2 ( kindly provided by L. Naldini, Sandoz (Novartis), Switzerland) and 10% FCS. 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 (kindly provided by GlaxoSmithKline, Rixensart, Belgium), 1 µg/ml PHA (Oxoide, Basel, Switzerland) and 1 × 106/ml irradiated (3000 rad) allogeneic PBMCs as feeder cells. Culture medium was checked daily and changed when required, and cells were stimulated every 20 d of culture.

**Soluble TCR production and measurements of TCR affinity**

The detailed procedure for the generation of high-affinity TCR Vα-chains upon structure-based design is available elsewhere (28, 29 and V. Zoete and O. Michielin, unpublished observations). Mutations were introduced into the wild-type (WT) TCR BV13.1 (patient LAU 155) DNA by PCR mutagenesis using the QuickChange mutagenesis kit (Strategene, La Jolla, CA) and confirmed by DNA sequencing. In brief, TCRα- (AV23.1) and TCRβ- (BV13.1) chains were subcloned by PCR separately into the pG7 vectors (Stratagene) and confirmed by DNA sequencing.

Expression of transduced TCRs was measured by flow cytometry on d5 posttransduction. T cell production and cell transduction were conducted in at least three independent experiments and produced comparable results, indicating 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-A2/2N–ESO–1.157–165 (SLLWITQA) multimers as described previously (28) and with PE-H- or FITC-conjugated Abs against Vβ13.1 (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 multimers. For dissociation experiments, T cells expressing an FACS/VSE multimer (BD Bioscience, 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 or variant TCRs were further enriched following sorting of multimer+ T cells using a FACSVantage SE machine (BD Bioscience) and included CD8 T cells expressing the Vβ49 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 with PE-labeled HLA-A2/2N–ESO–1.157–165 (SLLWITQA) multimers as described previously (28) and with PE- or FITC-conjugated Abs against Vβ13.1 (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 multimers. Flow cytometry analyses were performed on an LSR II flow cytometer (BD Biosciences, Biosciences, Genentech, CA), and data were analyzed using CellQuest software (Tampa, FL). FACS 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 multimer+ T cells using a FACSVantage SE machine (BD Bioscience) and included CD8 T cells expressing the Vβ49 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%).
**Confocal microscopy analysis**

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/493–503 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, Lauda-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 for segmentation and analysis. First, a journal has been made to count automatically the number of cells based on standard area of the nuclei 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 size 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 (n-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 Na3VO4. 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, Zug, Switzerland), anti–phospho-ERK1/2 (Cell Signaling Technology, BioConcept, Allschwil, Switzerland). Membranes were stripped in buffer containing 0.7% 2-ME, 2% SDS, and 0.06 M 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 × 10^6) for 24 h and stained with 1% Nonidet P-40, 1% lauryl maltoside, 50 mM Tris (pH 7.5), 140 mM NaCl, 10 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4. 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, Zug, Switzerland), anti–phospho-ERK1/2 (Cell Signaling Technology, BioConcept, Allschwil, Switzerland). Membranes were stripped in buffer containing 0.7% 2-ME, 2% SDS, and 0.06 M 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).

**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-clono1) 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-clono1 by only 4 aa residues, two within the CDR3a loop (T95Q and S96T) and two within the CDR3b 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-clono1 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 (V49, G50, A51) most 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 NA8 (A2/NY–ESO-1–) truncated into the irrelevant CMV peptide.

**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).

**Table I. Affinities of the A2/NY–ESO-1–specific TCR WT and its variants**

<table>
<thead>
<tr>
<th>CDR2</th>
<th>CDR3</th>
<th>Kd (μM)</th>
<th>koff (M⁻¹ sec⁻¹)</th>
<th>kcat (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (LAU155 BV13c1)</td>
<td>S</td>
<td>V</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>V49⁵</td>
<td>S</td>
<td>I</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>G50A⁴</td>
<td>S</td>
<td>V</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>A51E⁴</td>
<td>S</td>
<td>V</td>
<td>G</td>
<td>E</td>
</tr>
<tr>
<td>G50A+A51E⁴</td>
<td>S</td>
<td>V</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>A97L⁴</td>
<td>S</td>
<td>V</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>G50A+A51E+A97L⁴</td>
<td>S</td>
<td>V</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>1G4 (36)</td>
<td>S</td>
<td>V</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>wtc51 (modified)</td>
<td>S</td>
<td>V</td>
<td>A</td>
<td>I</td>
</tr>
</tbody>
</table>

⁴TCR variants, with amino acid replacements within CDR2β and/or CDR3β loops of the TCR BV13-clono1 (LAU 155) were rationally designed by a novel in silico structure-based approach [28, 29], V. Zoete, M. Irving, M. Ferber, D. Schmid, N. Rufer, and O. Michielin, manuscript in preparation. koff and kcat values were measured using SPR carried out with soluble constructs generated from TCR BV13-clono1 WT and its variants as detailed in Materials and Methods.

⁵TCR 1G4 (36) has two amino acid differences in CDR3β (A97N+A98T) and two in CDR3α (Q95T+T96S; not indicated) compared with WT TCR BV13-clono1. koff, kcat, and kcat values were measured by Chen et al. (36).

⁶The modified wtc51 TCR variant comprises the WT BV13-clono1 chain sequences with CDR2β amino acid replacements G50A, A51I, G52Q, and I53T, as described by Ref. 21, thus resulting in the drastic increase of its affinity toward pMHC, n.a, not applicable.
and A97L (CDR3β) substitutions for the pMHC complex by SPR. When compared with the WT TCR (\(K_D, 21.4 \text{ nM}\)), TCRs variants showed an incremental hierarchy in affinity from single mutants (A51E < G50A < A97L) to the double mutant G50A+A51E (\(K_D, 1.9 \mu \text{M}\)), and the triple mutant G50A+A51E+A97L (\(K_D, 0.9 \mu \text{M}; 23\)-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).
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 wt51 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. 1A) 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) allelogeneic HLA-A1 or-A3 multimers (Fig. 1A; data not shown).

We then assessed cell surface-bound multimer on-rates (Fig. 1B, 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 < wt51 (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 (k_{off}, min^{-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 (K_D) as well as with dissociation rates (k_{off}, 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 wt51 TCR variant of nanomolar range affinity exhibited the slowest multimer off-rates (with an average mean t_{1/2} of 62 min for CD8 cells). Of note, the natural TCC from which the WT TCR BV13-clono1 had been originally isolated (31) depicted slower off-rates than 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...
Levels of LAT and ERK phosphorylation in SUP-T1 and CD8 T cells expressing TCR variants. A, TCR-untransduced (Ø) 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 (unst 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 group. 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 assayed 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.

**FIGURE 3.** Levels of LAT and ERK phosphorylation in SUP-T1 and CD8 T cells expressing TCR variants. A, TCR-untransduced (Ø) 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 (unst 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 assayed 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.

Activation of ERK has been shown to be essential in mediating T cell function such as proliferation (40, 41). Therefore, we conducted a quantitative cytometric analysis of the proliferative response of T cells to T2 cells loaded with the NY–ESO-1157–165 peptide using CFSE as an indicator of cell division (Fig. 3D). In agreement with the stronger and more sustained MAPK activation, CD8 T cells transduced with the G50A+A51E mutant showed an increased proliferative capacity as compared with cells transduced with WT TCR. Conversely, reduced proliferation potential was found for T cells expressing V49I, correlating with the low levels of phospho-ERK expression in those cells. Finally, no differences in relative proliferation were observed for T cells expressing A51E, A97L, and G50A+A51E+A97L, consistent with the observation that levels of p-LAT and p-ERK1/2 for the A97L TCR variant, tested in addition, were not higher than for the WT TCR (data not shown).

**TCR variants of increased affinity reveal enhanced T cell functionality but reach a plateau of maximal activity**

To evaluate functional avidity and fine specificity of Ag recognition by our panel of transduced CD8 T cells, we conducted chromium-release assays to assess their ability to recognize target cells pulsed with graded concentrations of NY–ESO-1157–165 peptide (Fig. 4A). All transduced CD8 cells were able to recognize and lyse T2 target cells loaded with the cognate peptide but not with irrelevant peptides (i.e., FluMA, CMV pp65, or Melan-A/MART-1; data not shown). T cells transduced with V49I required more peptide than WT TCR-transduced CD8 T cells to achieve comparable and efficient lysis (Fig. 4B). The remaining transduced CD8 T cells fell into two distinct groups. Those in the first group, including WT, A51E, and 1G4 TCR variants, shared observed in SUP-T1 cells expressing G50A+A51E TCR mutant (Fig. 2) would also lead to an enhanced activation of downstream TCR signaling proteins such as LAT and ERK 1/2. Following multimer stimulation, Western blot analysis revealed a drastic reduction of ERK phosphorylation levels in both SUP-T1 and CD8 T cells expressing the V49I TCR mutant, highly contrasting with phospho-ERK levels found in WT and G50A+A51E-transduced T cells (Fig. 3A). Importantly, there were no differences in phosphorylation upon stimulation of the cells with the anti-TCR Ab OKT3, indicating that the TCR-mediated signaling machinery leading to MAPK activation was fully functional. A kinetic analysis revealed that LAT phosphorylation was rapid and transient, with maximal phosphorylation of residue Y171 being reached after only 1 min following specific stimulation (Fig. 3B, 3C). Strikingly, LAT phosphorylation was higher in primary CD8 T cells transduced with the G50A+A51E mutant than in cells expressing WT TCR. Also, increased basal levels of phosphorylation were already detectable in G50A+A51E-transduced CD8 T cells in the absence of multimer stimulation (Fig. 3C, insert). Finally, more rapid and sustained ERK2 phosphorylation was observed in G50A+A51E-transduced CD8 T cells, reaching up to a 7-fold increase when compared with cells expressing WT TCR (Fig. 3C).
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 reveal a robust correlation between multimeric TCR–pMHC binding off-rates (k_{off}, min⁻¹) and functional killing activity; there was a drastic reduction in killing function of T cells expressing the lowest binding affinity TCR (e.g., V49I), and, conversely, enhanced function in those cells of highest TCR affinity. Importantly, above a given TCR affinity threshold (delineated by the affinity of the A51E TCR variant, K_D < 7.1 μM), 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_{50b} 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 ≤ 1.9 μM) 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-1157–165 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

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**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<sup>−/−</sup>) loaded with graded concentrations of analog (SLLMWITQQA) NY-ESO-1<sub>157–165</sub> peptide. For each experiment, we included T2 cells pulsed with the irrelevant Melan-A<sub>26–35</sub> 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 (SLLMWITQQA) NY–ESO-1<sub>157–165</sub> peptide (data not shown).
variants are plotted against: 1) multimer off-rates (maximal target cell lysis) and TCR–pMHC binding avidity (multimer off-separating two distinct groups of TCR variants.

An analog NY–ESO-1157–165 peptide. Ag-specific lytic activity was assessed in a lymphocyte to target ratio of 10:1 in the presence of serial dilutions of the tumor peptides expressed by this cell line is likely to be excluded.

tumor associated Ags to which the endogenous T cell repertoire is nonresponsive (reviewed in Refs. 43, 44). The feasibility of TCR developed with the aim to induce immune reactivity toward defined melanin–A–specific TCRs also targeted normal tissues expressing the cognate Ag (46). These results underline not only the cytotoxic potency in vivo, but also the importance of the tissue distribution of tumor (self-) Ag expression. Therefore, much attention has been focused on the choice of Ag specificity (47). The cancer testis Ag NY–ESO-1 appears to be a preferred choice because its expression is found in melanoma and many other types of cancer cells but not in somatic adult tissues, with the exception of testis cells that do not express MHC molecules.

One highly promising approach toward the improvement of adoptive cell transfer cancer therapy utilizing TCR gene transfer is to modify TCR sequences to increase their affinity for cognate tumor Ag epitopes (43, 44). Recently, various strategies such as phage-display TCR library screens have led to the generation of 1G4 TCR variants with supraphysiological binding strength for the NY–ESO-1157–165 epitope of up to picomolar affinities (20, 21). Although some of the variants identified showed enhanced T cell function, the increase in affinity oftentimes also led to loss of target cell specificity (24). Interestingly, Robbins and colleagues (25) recently defined an upper affinity limit for these 1G4 TCR mutants in CD8 T cells that is compatible with specific Ag recognition and lies between 450 and 280 nM. At present, the major challenge is no longer to simply maximize the affinity of any given self–tumor-reactive TCRs but to finely tune and optimize TCR affinity and binding kinetics in a step-by-step approach to maximize T cell functionality. This implies an ability to precisely determine the impact of each optimized TCR variant on its binding to pMHC, downstream signaling, and subsequent T cell function.

In this study, we characterized a selected panel of TCR variants specific for the pMHC ligand A2/NY–ESO-1157–165 and derived from the original TCR BV13-clonol1 (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 ΔΔGbind value and for which titration ELISA revealed extremely low levels of binding to pMHC (data not shown). The second outlier assessed, the TCR wtc51, was adapted from Dunn et al. (21) and had the highest affinity of all TCRs characterized (Kd = 15 nM).

A major finding of this study is the remarkable correlation between monomeric soluble TCR–pMHC binding affinity (Kd) and multimeric TCR–pMHC dissociation kinetics on T cells (t1/2), 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 wtc51-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 the surface of T cells expressing those receptors. Yet it still remains unclear whether an increase in the t1/2 of the binding of the TCR to the A2/NY–ESO-1 complex would translate into a greater...
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) or negative (e.g., V49I and WT) changes in affinity (EC50) of CD8-transduced TCR WT and variants.

FIGURE 6. Melanoma cell killing by CD8 T cells transduced with TCR variants. A and B, Tumor reactivity for the melanoma cell lines Me 275 and Me 290 (A2+/NY–ESO-1+), and NA8-MEL (A2+/NY–ESO-1−) in the absence (A) or presence (B) of analog NY–ESO-1_{157-165} 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.
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 wtC51 TCR variant (K_D of 15 nM), which displayed both limited functional avidity (as measured by EC50) 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 antigen T cell response (Fig. 6D).

Recently, Chernov and coworkers (53) investigated the specific role of the CD8 coreceptor on a large panel of 2C TCR affinity variants specific for SIYR/K'. In their study, they measured the IL-2 release by transduced CD8 coreceptor-negative T cell hybridomas 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 TCR 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 EC50 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 V49I 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-clonol 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. Low T cell receptor specificity and avidity sufficient for in vitro proliferation or cytotoxicity to peptide-coated target cells but not for in vivo protection. J. Immunol. 149: 972–980.


AFFINITY AND AVIDITY OF NY-ESO-1–SPECIFIC TCR VARIANTS


Supplemental Figure 1. TCR-pMHC binding kinetics in SUP-T1 cells transduced with WT and variant TCRs. (A) TCR surface expression and specificity was evaluated in transduced SUP-T1 cells by flow cytometry analysis using anti-BV13 Ab and A2/NY-ESO-1_{157-165} multimers. Stainings of cells transduced with TCR 1G4 and wtc51 gave similar results (data not shown). To allow direct comparison between samples, we always used SUP-T1 cells expressing > 90% of transduced TCR BV13. (B) The rate of association of multimers on transduced SUP-T1 cells was measured as described in Materials and Methods. Background levels were assessed with SUP-T1 cells expressing an irrelevant Melan-A specific TCR. (C) After staining transduced SUP-T1 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 for a representative experiment and time for half maximal binding (t_{1/2}) was determined. (D) Average k_{off} values (dissociation constant of the pMHC from the TCR, min^{-1}) assessed during multimer dissociation assays and representative of nine independent experiments. Data were analyzed by unpaired two-sample t test. The dotted line was arbitrarily set at the k_{off} value obtained for WT TCR and allows direct comparison between the different transduced SUP-T1 cells. (E) Relationships between multimer off-rates (k_{off}, min^{-1}) and monomeric affinity (K_{D}, μM) or off-rates (k_{off}, s^{-1}) as measured by SPR, expressed as log-log fitting straight lines.