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

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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-1157–165–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 (Kd < ~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.
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 clones 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 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 avidity 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 avidity 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 N8), T2-A2 (TAP-deficient lymphoblastoid cell line transfected with HLA-A*0201), CIR-WT, and CIR-CDS**mut** cells were cultured in RPMI 1640 (In Vitrogen, 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 later use or expanded posttransduction in RPMI 1640 medium supplemented with 8% human serum, 150 U/ml recombinant human IL-2 (a gift from GlaxoSmithKline, Rixensart, Belgium), 1 μg/ml PHA (Oxoid, Basel, Switzerland) and 1 × 10⁶/mi irradiated (3000 rad) allogeneic PBMCs as feeder cells. Culture medium was checked daily and changed when required, and cells were stimulated every 10 d of culture.

**Soluble TCR production and measurements of TCR avidity**

The detailed procedure for the generation of high-affinity TCR Vβ-chains upon structure-based design is available elsewhere (28, 29, and V. Zoete and L. Naldini, 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, TCRβ (AV23.1) and TCRα (BV13.1) chains were subcloned by PCR separately into the pGEMT vector (Beckton Dickinson, San Diego, CA). Cross-reactivity of TCR variants was assessed by multimer staining and/or with PE- or FITC-conjugated anti-CD8 mAb, washed once, and resuspended in buffer. An aliquot (corresponding to time t₀) was taken and analyzed by flow cytometry. Mean fluorescence intensity (MFI) data were collected from the TCR. Mean fluorescence intensity (MFI) data were collected from the TCR. The full length codon-optimized TCR AV23.1 and TCR BV13.1 were transfected with HLA-A*0201/NY–ESO-1 multimers (SLLMWITQA) and the LV transfer vector, envelope, and packaging plasmids (pR8.9, pMD2-2SV-G, and pMDL/pRRE). 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 × 10⁶/ml CD8 T cells were transfected with pretransduced polyethyleneparticles (1 μg/ml) and transduced with concentrated LV supernatant. Expression of transduced TCRs was measured by flow cytometry on d 5 posttransduction. TCR expression 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-A*0201/NY–ESO-1 multimers as described previously (30) and/or with PE- or FITC-conjugated anti-CD8 mAb, washed once, and resuspended in buffer. An aliquot (corresponding to time t₀) was taken and analyzed by flow cytometry. Expression of each optimized TCR variant on the TCR–pMHC interaction and that above or below this window, T cells may not develop productive function. The Journal of Immunology 4937
Confocal microscopy analysis

TCR-transduced SUP-T1 cells were washed once in RPMI 1640 medium and pellets resuspended at 3 x 10^6 density/ml. Staining was performed either with PE-labeled A2/NY-ESO-157-165 or A2/CMV493-503 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 autofluorescence of the cell membrane fluorescence. "m" journal measured the surface of the cell and cut its border by an erosion of 2 (interactive pen display, Cintiq 21UX, Wacom, Saitama, Japan), a second with Hoechst 33342. Then, after a manual selection around cell membrane for segmentation and analysis. First, a journal has been made to count autofluorescence of the cell membrane fluorescence. "m" journal measured the surface of the cell and cut its border by an erosion of 2 (interactive pen display, Cintiq 21UX, Wacom, Saitama, Japan), a second with Hoechst 33342. Then, after a manual selection around cell membrane for segmentation and analysis. First, a journal has been made to count autofluorescence of the cell membrane fluorescence. "m" journal measured the surface of the cell and cut its border by an erosion of 2 (interactive pen display, Cintiq 21UX, Wacom, Saitama, Japan), a second with Hoechst 33342. Then, after a manual selection around cell membrane for segmentation and analysis. First, a journal has been made to count autofluorescence of the cell membrane fluorescence.

Western blot analysis

Typically, 2 x 10^6 CD8 T cells per lane or 1 x 10^6 SUP-T1 cells per lane were used for biochemical analyses. 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-157-165 multimer (10 µg/mL) at identical 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 phenylmethyl sulfonyl fluoride, and 1 mM Na3VO4. Postnuclear supernatants were subjected to immunoblotting. Proteins were separated by SDS-PAGE followed by electrophoretic transfer to nitrocellulose membranes. Subsequently, membranes were probed with the following Abs: anti–phospho-tyrosine 4G10 (Upstate/Millipore, Zug, Switzerland), anti–phospho-LAT (LAT Y-171, and 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 x 10^6) transduced with WT or variant TCRs were incubated with T2 target cells (0.5 x 10^6) pulsed with analog NY-ESO-157-165 peptide (0.01 µg/mL, SLLMWTIQA) or CMV pp65 peptide (1 µg/mL, NLVPMVQTQ) 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 x 10^5 cells were collected and analyzed on an LSR II flow cytometer (BD Biosciences). The percentage of CD8 T cells proliferating (CFSE(+) in response to NY-ESO-1-specific stimulation was estimated from the proportion of corresponding CFSE-labeled cells (CFSE(+) stimulated with the irrelevant CMV peptide.

Lytic activity and Ag recognition was assessed functionally in 4 h [51Cr]-release assays using (1) T2 target cells or CIR target cells that expressed either WT or CD8-null HLA-A2 (HLA-A2/TAP(-)) pulsed with serial dilutions of analog NY-ESO-157-165 peptide [SLLMWITQA (34)], or Melan-A26-35 A27L 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-157-165 peptide. The percentage of specific lysis was calculated as 100 x (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-A0201/ NY-ESO-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 CD3ζ loop (T95Q and S96T) and two within the CD3β 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 CD2β (V49, G50, A51) and CD3β (A97) (Table I). Residues V49, G50, and A51 mostly interact with the HLA-A0201 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 (CD2β), and A97L mutations. The results of these in silico calculations are briefly summarized in Table I.

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

<table>
<thead>
<tr>
<th>TCR BV13</th>
<th>1G4 (36)</th>
<th>WT (LAU155 BV13clono1)</th>
<th>V49A</th>
<th>G50A</th>
<th>A51E</th>
<th>G50A+A51E</th>
<th>A97L</th>
<th>G50A+A51E+A97L</th>
<th>wt51 (modified)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n.d.</td>
<td>1.2 x 10^6</td>
<td>1.2 x 10^6</td>
<td>2.0 x 10^6</td>
<td>2.0 x 10^6</td>
<td>2.0 x 10^6</td>
<td>2.0 x 10^6</td>
<td>2.0 x 10^6</td>
<td>2.0 x 10^6</td>
<td>2.0 x 10^6</td>
</tr>
<tr>
<td>KD (µM)</td>
<td>3.8</td>
<td>0.013</td>
<td>0.013</td>
<td>0.013</td>
<td>0.013</td>
<td>0.013</td>
<td>0.013</td>
<td>0.013</td>
<td>0.013</td>
</tr>
<tr>
<td>κoff (s^-1)</td>
<td>1.1 x 10^6</td>
<td>1.1 x 10^6</td>
<td>1.1 x 10^6</td>
<td>1.1 x 10^6</td>
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<td>1.1 x 10^6</td>
<td>1.1 x 10^6</td>
</tr>
<tr>
<td>κon (M^-1 s^-1)</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
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<td>1.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

aT1 cells, with amino acid replacements within CDR2 on, and/or CDR3 loops of the TCR BV13clono1 (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]. KD, κoff, and κon values were measured using SPR carried out with soluble constructs generated from TCR BV13clono1 WT and its variants as detailed in Materials and Methods.

bTCR 1G4 (36) has two amino acid differences in CDR3β (A97N+A98T) and two in CDR3α (Q95T+T96S; not indicated) compared with WT TCR BV13clono1. KD, κoff, and κon values were measured by Chen et al. (36).

cThe modified wt51 TCR variant comprises the WT BV13clono1 chain sequences with CD2β 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 μM), TCRs variants showed an incremental hierarchy in affinity from single mutants (A51E $< G50A < A97L$) to the double mutant G50A+A51E ($K_D$, 1.9 μM), and the triple mutant G50A+A51E+A97L ($K_D$, 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).
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. 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 < 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 ($k_{	ext{off}}$, 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 ($k_{	ext{off}}$, 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, IG4, 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 $t_{1/2}$ 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 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...
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. 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.

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 using CFSE as an indicator of cell division (Fig. 4D). 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, correlating with the low levels of phosphorylation 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).

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 a measure 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 phosphorylation 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.
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 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 (delimited 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 avidities.

**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
variants are plotted against: 1) multimer off-rates (rates) or affinity (SPR analysis). Functional avidities of CD8-transduced TCR
maximal target cell lysis) and TCR–pMHC binding avidity (multimer off-
separating two distinct groups of TCR variants.

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
assessed using cell line C1R expressing either WT (opened squares) or

Discussion
Adoptive transfer of TCR gene-modified T cells has been recently
developed with the aim to induce immune reactivity toward defined
tumor-associated Ags to which the endogenous T cell repertoire is
nonresponsive (reviewed in Refs. 43, 44). The feasibility of TCR
gene transfer was initially demonstrated in a phase I clinical trial
whereby patients with melanoma received autologous PBLs trans-
duced with a specific TCR against the differentiation Ag Melan-A/
MART-1 (45). More recently, Johnson and colleagues (46) con-
ducted another extensive study in patients with metastatic melanoma
-treated with genetically engineered T lymphocytes and demon-
strated persistence in the blood of the transduced cells as well as
objective cancer regressions. However, in addition, patients ex-
hibited toxicity with destruction of normal melanocytes in the skin,
eye, and ear, indicating that T cells expressing highly reactive
Melan-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 screenings 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). Al-
though 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-clonol (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 amino
acid residues of defined importance for the TCR–pMHC
interaction and binding; 2) structure-based design of correspond-
ing 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 hierar-
chy 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 ΔΔ∆G binding 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 multi-
meric 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 par-
pecially 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
T 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 TCR–pMHC binding parameters (such as affinity, avidity, and clustering), downstream signaling pathways, and cellular functionality (T cell proliferation and target cell killing).

Major efforts have been made to characterize and identify specific binding parameters ($K_D$ or $t_{1/2}$) that control T cell activation (reviewed in Refs. 8, 48). Kinetic models of TCR–ligand interaction propose that functional potency is primarily determined by the duration of the TCR–pMHC interaction (49, 50) because sufficiently long dissociation rates may be required for completion of intracellular signaling cascades and subsequent T cell activation. The serial triggering hypothesis (51), however, suggests that dissociation rates need to be sufficiently short to allow an optimal dwell time of interaction between the TCR and pMHC complex so that multiple TCRs on the cell surface can sample the pMHC complex for efficient T cell activation (52). These models are not mutually exclusive, and indeed our results are consistent with both of them. First of all, we observed that TCR/multimer off-rates directly correlated with functional avidity (as determined by the peptide concentration required to achieve half-maximal target lysis). Thus, slower dissociation rates were found for the transduced CD8 T cells of relatively high functional avidity (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
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 wt51 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 antigen 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^D. 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 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, a functional and/or secretarial help and helpful discussions. We also thank the technical and/or secretarial help and helpful discussions. We also thank the "Vital-T project of the Swiss Institute of Bioinformatics (Lausanne, Switzerland) for providing the computational resources.

Disclosures

The authors have no financial conflicts of interest.

References
