Interplay between T Cell Receptor Binding Kinetics and the Level of Cognate Peptide Presented by Major Histocompatibility Complexes Governs CD8⁺ T Cell Responsiveness*

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Background: Adaptive immunity requires T cell receptor (TCR) recognition of antigenic peptide in complex with major histocompatibility complexes (pMHC).

Results: Both TCR/pMHC binding kinetics and the amount of interacting cognate pMHC contribute to CD8⁺ T cell activation.

Conclusion: Intermediate TCR/pMHC binding parameters confer maximum biological responses.

Significance: Elucidating the mechanism of T cell activation is critical for vaccine and immunotherapeutic development.

Through a rational design approach, we generated a panel of HLA-A*0201/NY-ESO-1157–165-specific T cell receptors (TCR) with increasing affinities of up to 150-fold from the wild-type TCR. Using these TCR variants which extend just beyond the natural affinity range, along with an extreme supraphysiologic one having 1400-fold enhanced affinity, and a low-binding one, we sought to determine the effect of TCR binding properties along with cognate peptide concentration on CD8⁺ T cell responsiveness. Major histocompatibility complexes (MHC) expressed on the surface of various antigen presenting cells were peptide-pulsed and used to stimulate human CD8⁺ T cells expressing TCR within a dissociation constant (KD) range of ~1–5 μM. Under these same conditions there was a gradual attenuation in activity for supraphysiologic affinity TCR with KD < ~1 μM, irrespective of CD8 co-engagement and of half-life (t1/2 = ln 2/koff) values. With increased peptide concentration, however, the activity levels of CD8⁺ T cells expressing supraphysiologic affinity TCR were gradually restored. Together our data support the productive hit rate model of T cell activation arguing that it is not the absolute number of TCR/pMHC complexes formed at equilibrium, but rather their productive turnover, that controls levels of biological activity. Our findings have important implications for various immunotherapies under development such as adoptive cell transfer of TCR-engineered CD8⁺ T cells, as well as for peptide vaccination strategies.

CD8⁺ T cells play a critical role in protective immune responses by T cell receptor (TCR)5-mediated recognition of infected or transformed cells displaying antigenic peptide in complex with Class I major histocompatibility complexes (MHC). TCR/pMHC interactions typically comprise fast kinetics and weak affinity with dissociation constants (KD), defined at equilibrium as the rate of dissociation divided by the rate of association (KD = koff/kon), ranging from 1–100 μM (1, 2). These binding properties, shaped during thymic selection, are necessary to enable TCR to avoid autoreactive responses against self-pMHC and to effectively trigger the T cell once a rare antigenic complex has been encountered (3, 4). Because TCR specific for MHC-presented peptides of tumoral origin (mostly self-proteins) tend to be of weaker affinity than TCR against peptides of pathogenic origin (5), it is widely accepted that genetically engineered CD8⁺ T cells expressing affinity-optimized TCR will be of clinical benefit to cancer patients treated by adoptive cell transfer (6).

Extensive research has been undertaken to elucidate the correlates of TCR/pMHC binding parameters and T cell activation

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5 The abbreviations used are: TCR, T cell receptor; MHC, major histocompatibility complex; MM-GBSA, Molecular Mechanics-Generalized Born Surface Area; APC, antigen-presenting cell.
(7), an understanding of which has implications for various immunotherapies under development against cancer, autoimmune disease, viral infection, and allergies (8). Two main models of activation have emerged, both of which are debated. The affinity model, also known as equilibrium model, (9, 10) proposes that the total number of TCR bound to pMHC at equilibrium regulates activity (i.e. receptor occupancy). The half-life model, also referred to as the productive hit rate model or the turnover model, the one gaining broader acceptance, stipulates that for T cell activation to occur the receptor and ligand must stay bound for sufficiently long duration to enable biochemical responses necessary for productive signaling, but must release quickly enough such that other TCR within the synapse have the opportunity to sample the rare antigenic pMHC and thus amplify signaling cascades (i.e. have an optimal dwell time and enable serial triggering) (11–15).

We have developed a rational in silico design method for optimizing TCR/pMHC interactions based on Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) free energy calculations and TCR/pMHC crystal structures (16, 17). Previously we applied our design strategy to the β-chain of an HLA-A*0201-restricted NY-ESO-1\textsubscript{157–165} -specific TCR (TCR BC1), derived from a long-surviving melanoma patient (18), and developed variants with up to 24-fold increase in affinity (19). Examination of CD8\textsuperscript{+} T cells lentivirally transduced to express one of the affinity-optimized TCR (\(K_D = 1.91 \mu M, t_{1/2} = 15.4 \text{ s}\)) revealed enhanced TCR-mediated signaling and proliferation as compared with the wild-type (WT) TCR (\(K_D = 21.4 \mu M, t_{1/2} = 3 \text{ s}\)). Comparison of cytotoxicity for the panel of TCR revealed a functional plateau in killing for CD8\textsuperscript{+} T cells expressing the binding-enhanced TCR, but an extreme supersphysiologic TCR (\(K_D = 15 \text{ nM}, t_{1/2} = 553 \text{ s}\)) conferred markedly reduced cytotoxicity, suggesting the presence of a TCR affinity threshold delimiting maximum CD8\textsuperscript{+} T cell function. These intriguing findings prompted us to investigate whether low supersphysiologic TCR having affinity just beyond the natural range would maintain or break this apparent threshold, or if, like the extreme supersphysiologic TCR, they would also attenuate function.

Here, to further explore the effect of TCR/pMHC binding parameters on CD8\textsuperscript{+} T cell responsiveness we applied our modeling strategy to the BC1 α-chain and measured biological activity levels at different peptide dosage of cell-surface displayed MHC molecules. By rationally combining optimized α- and β-chains we developed HLA-A*0201/NY-ESO-1\textsubscript{157–165} -specific variants with up to 150-fold increase in affinity from WT. At intermediate peptide loading of antigen-presenting cells (APCs) we observed maximum T cell function, including cytokine/chemokine secretion, cytotoxicity and Ca\textsuperscript{2+} flux, for TCR with \(K_D \sim 1–5 \mu M\). Just beyond the upper limit of the natural affinity range, however, at \(K_D \sim 1 \mu M\), all biological activities were progressively attenuated, regardless of CD8 co-engagement or of half-life. In contrast, at increased peptide-dosage activity levels were gradually restored for CD8\textsuperscript{+} T cells expressing supersphysiologic affinity TCR. Overall our data show that the maximum T cell response occurs at intermediate binding parameters and they support the productive hit rate model for T cell activation.

**EXPERIMENTAL PROCEDURES**

**TCR Modeling**—As previously described (16, 17, 20), the MM-GBSA approach was applied to TCR BC1, to estimate the contribution of individual amino acids to the binding energy with A2/NY-ESO\textsubscript{157–165} and identify amino acid replacements in the TCR that would enhance or disrupt (in the case of V49I) this interaction. Binding free energy estimations were performed using the crystal structure for the closely related TCR 1G4 (the four amino acid differences between 1G4 and BC1 are αT95Q, αS96T, βN97A, & βT98A, respectively) in complex with A2/NY-ESO-1\textsubscript{157–165} (PDB ID 2BNR) (21). All molecular graphics images were produced using the UCSF Chimera package (22).

**Protein Expression in HEK-293 Cells and Purification**—The α- and β-chain of TCR BC1 were cloned separately into pHYK8 under the control of a CMV promoter. Following the strategy of Chang et al., (23), heterodimeric chain pairing was facilitated with an acidic-basic zipper. The TCR β-chain was truncated after position Cys-242 and replaced by a flexible linker region, a thrombin site, an acidic zipper and a His tag (SFEDLVPRGST-TPASAQLEKALEKENAQLEWELALELQALQAEKELAQTGHH-HHHH). The α-chain was truncated after Cys-209 and replaced by a linker region, a thrombin site, and a basic zipper (SSADL-VPRGSTAPSAQLEKALKKAKLKKNLKWKLELQALKALKK-AL-AQ). Soluble TCR was produced by co-transfection of the plasmids with linear 25 kDa polyethylenimine into HEK-293 cells. The transfected cells were cultured in suspension for 5–7 days in Pro293 CDM medium (Lonza) supplemented with 4 mM valproic acid to minimize acidification. Culture supernatant was collected by centrifugation, and the TCR purified using Ni-NTA agarose (Qiagen) following the manufacturer’s suggestions.

**Protein Refolding from Bacterial Inclusion Bodies and Purification**—BL21(DE3)pLys bacterial cells were used to produce TCR α- and β-chain (cloned into pGEMT7) as inclusion bodies, which were solubilized and refolded by dialysis as previously described (24). The TCR were then concentrated and filtered prior to fast protein liquid chromatography His-tag purification with Ni\textsuperscript{2+} immobilized metal chelating Sepharose (GE Healthcare) and imidazole elution. Prior to SPR analysis the samples were concentrated on 10kDa MWCO spin filters (Millipore) and gel-filtered into HBS-EP buffer (10 mM HEPES, pH7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v P20 surfactant), using a S200 column to remove aggregate. Biotinylated A2/NY-ESO\textsubscript{157–165} was prepared as previously described (25).

**Surface Plasmon Resonance**—SPR was performed on a BIAcore 3000 and SA-coated, CM5 sensor chips (BIAcore, GE Healthcare) at 25 °C. Flow cells were loaded with 200 response units of biotinylated pMHC at a rate of 10 µl/min for uniform distribution. Free sites on streptavidin (SA) were biotin-blocked and a reference cell was used as a control against changes in bulk refractive index upon injection of sample solution. For kinetic analysis, six to eight serial dilutions of TCR were injected over the loaded chip at 50–100 µl/min. Data are from one experiment of at least two independent experiments, conducted in duplicate or in triplicate, giving the best chi\textsuperscript{2} values. The \(k_{on}\) and \(k_{off}\) values for each TCR were calculated...
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assuming 1:1 Langmuir binding, and data were analyzed using BLAevaluation 4.1 and a global fit algorithm. $K_D$ was calculated by $k_{off}/k_{on}$.

**Lentivirus Production and Cell Transduction Efficiency—** Full-length codon optimized DNA encoding the TCR $\alpha$- and $\beta$-chains were cloned in the pRRL third generation lentiviral vector (26) as a hPGK- AV23.1-IRES- BV13.1 construct (19). Lentiviral vectors were produced by transient transfection of 293T cells using a standardized protocol for calcium phosphate precipitation (27). The lentiviral supernatant was concentrated by ultracentrifugation. Human CD8$^+$ T cells, isolated by negative depletion (MACS, Miltenyi Biotech), or SUP-T1 cells (28), were transferred to polybrene-treated plates, at $2 \times 10^6$ and $1 \times 10^6$ cells per well, respectively. The cells were transduced with concentrated lentiviral supernatant and expression of the introduced TCR measured 5 days later by fluorescent multimer staining. The number of copies of lentivirus stably integrated was measured by real-time PCR using genomic DNA extracted from $1 \times 10^6$ transduced CD8$^+$ T cells or SUP-T1 cells using Puregene Core Kit A (Qiagen) along with primers and TaqMan probes against the viral-specific gag sequence (provided by D. Trono, EPFL, Switzerland). The analysis was conducted on a 7900HT sequence detector machine, and data were analyzed using SDS7900HT software (Applied Biosystems). Oligos specific for the albumin gene were used to normalize the quantity of genomic DNA. Lentiviral integration was more or less equivalent for each TCR variant within each type of transduced cell; SUP-T1 cells ranged from 8–10 lentivirus copies/cell and CD8$^+$ T cells ranged from 1–2 copies/cell (data not shown).

**Cytometric Analysis—** SUP-T1 and CD8$^+$ T cells expressing the different TCR were stained with PE-labeled multimers as described (19). Cross-reactivity of the TCR variants was assessed by staining transduced SUP-T1 cells with a panel of 14 different PE-labeled multimers, for 1 h at 4 °C. Flow cytometry was conducted on an LSRII flow cytometer (BD Biosciences), and data were analyzed using CellQuest, FCS Express (De Novo Software), or FlowJo software (Tree Star, Ashland, OR). All staining experiments were conducted in duplicate and were replicates a minimum of two times.

**Multimer Dissociation Measurements—** Multimer dissociation measurements were performed on TCR transduced SUP-T1 cells with PE-labeled A2/NY-ESO$^{157-165}$ multimer (2 $\mu$g/ml) as described previously (19). These measurements were independently conducted at least three times for the TCR variants.

**ELISA—** Biotinylated A2/NY-ESO$^{157-165}$ complexes were captured on SA-coated plates (96 well, high-binding plates, Corning Life Sciences) blocked with 2% BSA in Tris-buffered saline (TBS, pH 7.4). Plates were washed between each step with TBS, 0.1% Tw. Free sites on SA were biotin-blocked subsequent to 1.5 h incubation at room temperature with soluble TCR in TBS, 1% BSA, 0.1% Tween. Bound TCR was detected with anti-$\beta$ chain TCR mAb (TCR 1151, Thermo Scientific, Rockford, IL), diluted 1/1500 in TBS, 1% BSA, 0.1% Tween, followed by HRP-conjugated-goat-anti-mouse IgG-Ab (Thermo Scientific), diluted 1/1500 in TBS, 0.1% Tween, and HRP detection with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in a citric acid-phosphate buffer containing H$_2$O$_2$. Plate readings were taken after 30 min. at $A_{405-490}$. The ELISA for each TCR was repeated at least three times.

**Cell Lines and Primary CD8$^+$ Lymphocytes—** The culture and propagation of SUP-T1 cells (a human T cell lymphoblastic lymphoma), CIR cells (CIR/A2 cells which were transfected to express HLA-A2, and CIR/A2$^{27-228}$ cells, which were transfected to express the CD8-binding deficient HLA-A2 D227K, T228A variant), T2 cells (A2$^+$/TAP$^->$), melanoma cell lines Me275 (A2$^+/$/NY-ESO157–165$^+$), Me290 (A2$^+$/NY-ESO157–165$^+$), and NA8 (A2$^+$/NY-ESO157–165$^+$), and lentiviral transduced CD8$^+$ T lymphocytes, was performed as described (19).

**Chromium Release, CD107a Expression, and Apoptosis Detection Assays—** Lytic activity of transduced T cells was assessed in 4 h $^{51}$Cr release assays. The assays were conducted using $^{51}$Cr-labeled T2 target cells, as well as the melanoma cell lines Me275, Me290, and NA8, in the presence or absence of graded concentrations of NY-ESO157–165 peptide analog C165A (SLLMWITQ). Analog peptide was used in all functional assays because the terminal Cys of the native peptide is redox susceptible and thus may form disulfide bridges with proteins in the culture medium (29). An effector to target ratio (E:T) of 10:1 was used unless stated otherwise. The percentage of specific lysis was calculated as: $100 \times$ (experimental-spontaneous release)/(total-spontaneous release). For the killing of T2 cells graded concentrations of NY-ESO157–165 peptide were used to obtain the half-maximum effective concentration (EC$_{50}$). For the melanoma cell lines, data are calculated as a ratio of the % specific lysis obtained without adding peptide divided by the % specific lysis obtained after adding 1 $\mu$M NY-ESO157–165 peptide. As a measure of T cell degranulation, CD107a-PE-Cy5 stainings were conducted on transduced T cells stimulated with NY-ESO157–165 peptide pulsed CIR/A2 and CIR/A2$^{27-228}$ (E:T ratio 2.5:1) cells. The anti-CD107a mAb was added for the duration of stimulation (1 h at 37 °C) and analyzed by flow cytometry. Results are shown as the % of cells that underwent degranulation. Chromium release assays and CD107a expression assays were conducted independently at least five times for each TCR variant. The apoptotic levels of TCR-transduced CD8$^+$ T cells were assessed after 6 h culture at 37 °C under 5% CO$_2$, both without and with serially titrated tetramer, by AnnexinV-FITC staining and cytometric analysis.

**Calcium Flux Assay—** The APCs CIR/A2 and CIR/A2$^{27-228}$ were pulsed side-by-side with graded concentrations of NY-ESO157–165 peptide for 1h incubation at 37 °C under 5% CO$_2$, followed by overnight incubation at 26 °C. The transduced CD8$^+$ T cells were loaded with 0.2 $\mu$M Indo 1-AM for 30 min at 37 °C prior to flow cytometric analysis. Both the APC and T cells were washed and intracellular Ca$^{2+}$ elevation assessed upon engagement of 50,000 T cells with 150,000 APC (E:T ratio of 1:3) following a 10 s co-sedimentation by centrifugation at 1400 rpm. Cells were kept at 37 °C during Ca$^{2+}$ flux analysis on a LSR II flow cytometer. Upon UV excitation, Indo 1-AM emission was measured at 405 nm/525 nm, reflecting the changes in intracellular Ca$^{2+}$ concentration. Data were recorded over 5 min. for each peptide concentration. Ionomycin stimulation of the T cells at the end of each experiment was used as a positive control (not shown). Data were analyzed using FlowJo Kinetics Module software (Tree Star). The cal-
cium flux assay was independently conducted at least twice for each TCR variant.

**Cytoxin and Chemokine Secretion Assay**—Both TCR-transduced and non-transduced CD8+ T cells, were stimulated for up to 40 h with irradiated T2 target cells in the presence or absence of graded concentrations of NY-ESO157–165 peptide. Cytokine (IL-2, 4, 6 & 10, IFN-γ, and TNF-α) and chemokine (IL-8, CCL5, CXCL9, CCL2, and CXCL10) content were measured by flow cytometry using Cytometric Bead Array kits (BD Biosciences) according to the manufacturer’s instructions. Data were analyzed with FCAP Array v1.0.2 software (SoftFlow Inc.). Secretion levels were measured for all four independent stimulation experiments.

**RESULTS**

**Binding Properties of Rationally Designed BC1 TCR Variants**—Previously, using the crystal structure of the near sequence identical TCR 1G4 in complex with A2/NY-ESO-157–165 (PDB ID 2BNR) (21), we rationally designed four β-chain BC1 TCR variants to incrementally enhance affinity (K_D, G50A > A97L > DM-β > TM-β) up to 24-fold, as measured by surface plasmon resonance (SPR), from the WT TCR (Fig. 1, A and B). According to our in silico modeling, gain in affinity for the variants resulted from increased contact with A2, and in the case of A97L, also through direct peptide contact (Fig. 1C). In addition, we developed a subphysiologic, weak-binding TCR (V49I), and an extreme supraphysiologic affinity TCR variant (wtc51m) based on one previously identified by phage-display screening (30) (Fig. 1D). Here we sought to further increase BC1 TCR affinity through modification of its α-chain.

To allow rapid TCR screening we established a mammalian cell expression system for producing soluble TCR, and compared the binding of 8 single amino acid replacement variants with pMHC by ELISA (Fig. 1D). The titration curves for the TCR reveal the remarkable specificity and sensitivity of TCR/pMHC interactions as the conservative replacements S52T, S53W, was paired with two of the previously optimized β-chains to generate the triple mutant and quadruple mutant TCR TM-α and QM-α with K_D values of 0.4 and 0.14 μM, respectively (Fig. 1A). All amino acid positions targeted for replacement in the TCR are depicted in Fig. 1E.

The calculated changes in binding free energy (ΔΔG) ranged between +1.9 to −16.14 kcal/mol (supplemental Fig. S1A) for the TCR variants which correlated with K_D measured by SPR (R² = 0.85; supplemental Fig. S1B). Primary CD8+ T cells and SUP-T1 cells were lentivirally transduced for cell surface expression of the different TCR. As shown by A2/NY-ESO157–165 multimer staining, the TCR were expressed at similar levels on the respective cell-types (except for the subphysiologic TCR V49I) (supplemental Fig. S2, A and B). Multimer dissociation experiments indicated half-lives that followed the same binding hierarchy as both TCR/pMHC affinity and MM-GBSA calculated changes in binding free energy (Fig. 1F and supplemental Fig. S1B).

**Specificity of Soluble and Cell Surface Displayed TCR Variants**—Because some affinity-enhanced TCR bind non-cognate pMHC and can be autoreactive (31–34) we scrutinized the specificity of our rationally designed TCR. First, we tested the binding of soluble TCR by ELISA with plate-immobilized A2/NY-ESO157–165 complexes containing Ala substituted NY-ESO157–165 Peptides. All of the TCR, including the extreme supraphysiologic affinity TCR wtc51m, exhibited similar binding patterns (Fig. 1G) with peptide residues M160, W161, I162, and Q164 being critical contact residues. This is consistent with structural analyses of 1G4 and its high affinity TCR variants (35). No binding to non-cognate pMHC was detected for any of the soluble TCR variants (data now shown). Second, we utilized a panel of 14 different multimers, comprising a range of different HLA-I alleles and peptides, to assess nonspecific binding by TCR-transduced cells and observed no differences from the WT TCR (supplemental Fig. S3). Thus, the affinity-enhanced TCR variants, both soluble and cell surface displayed, are ligand-specific.

**Cytokine and Chemokine Production by TCR-transduced CD8+ T Cells**—To assess the impact of TCR binding parameters on cytokine and chemokine production we incubated non-transduced and TCR-transduced CD8+ T cells with T2 cells pulsed with intermediate NY-ESO157–165 peptide dosage (1 μM, Fig. 2 and supplemental Fig. S4). For all lymphokines produced in response to TCR-mediated activation, including IFN-γ, TNF-α, II2, IL4, and IL8 (Fig. 2, A–E), secretion reached a maximum for cells expressing TCR G50A, A97L, and DM-β (K_D ~1–5 μM), and sometimes for the borderline supraphysiologic affinity TCR TM-β (K_D = 0.91 μM; Fig. 1A), and gradually decreased for CD8+ T cells expressing higher affinity TCR. This trend was also observed as early as 4 h (data not shown), and at later time points (20 h and 40 h; supplemental Fig. S4). Under the same conditions these molecules were not produced by non-transduced bulk CD8+ T cells but IFN-γ was detectable at a low background level that did not change over time (Fig. 2F). In all experiments the lowest secretion levels were for cells expressing the weakest-binding TCR V49I, and the highest affinity TCR wtc51m. Notably, unlike altered peptide ligands (APLs) that can lead to heteroclitic T cell responses (36), the panel of CD8+ T cells shared a uniform secretion profile.

**Cytotoxicity and Apoptosis of TCR Transduced CD8+ T Cells**—We assessed the cytolytic capacity of CD8+ T cells expressing the different affinity TCR against the melanoma tumor cell lines, Me290 and Me275, which are HLA-A2+ and naturally present NY-ESO157–165 (Fig. 3, A and B). Killing assays were conducted in both the absence and in the presence of intermediate dosage of exogenous peptide (1 μM) and data are presented as a killing efficacy ratio. For Me290 (Fig. 3A), CD8+ T cells expressing TCR G50, A97L, DM-β, and TM-β kill well-above WT levels in the absence of peptide-pulsing, but for the weak binding TCR V49I (the ratio nears zero) killing only occurs if cells have been pulsed. CD8+ T cells expressing the WT TCR and the highest affinity TCR, QM-α, and wtc51m, showed similar cytolytic capacity against Me290. For Me275, whereas, CD8+ T cells expressing the highest affinity TCR killed below WT levels (Fig. 3B). There was little or no killing of
the NY-ESO melanoma cell line NA8 by CD8 T cells expressing the TCR variants (Fig. 3C).

To confirm these results, as well as to explore the role of CD8 co-engagement in cytotoxicity, the percentage of CD107a up-regulation, (LAMP-1, a marker of degranulation) (37), was measured upon stimulation with NY-ESO,157–165 peptide-pulsed C1R/A2 cells (Fig. 3D), and with peptide-pulsed C1R/A2,227–228 cells (expressing mutated HLA-A2 which cannot be engaged by co-receptor CD8; Fig. 3E). The best-performing TCR were G50A, A97L and DM-β. In the absence of CD8 co-engagement the overall per-
percentage expression of CD107a was lower for all of the TCR variants, and CD8+ T cells expressing TCR with $K_D < 5 \mu M$ were less dependent on CD8 for their activity (Fig. 3F).

Lastly we sought to ensure that the attenuated activity of cells expressing the higher affinity TCR ($K_D < \sim 1 \mu M$) was not due to higher levels of apoptosis upon stimulation, nor to fratricide.
Cell death of the transduced CD8\(^+\) T cells themselves was measured in the presence of soluble pMHC tetramer as well as under normal culture conditions (Fig. 3, G and H). Under stimulatory conditions we measured the highest levels of apoptosis for CD8\(^+\) T cells expressing TCR G50A, A97L, and DM-\(\beta\). Thus, the CD8\(^+\) T cells that were the most activated in response to intermediate peptide dosage of APCs also underwent the highest levels of activation-induced cell death. Under normal culture conditions there were no differences in apoptosis among the CD8\(^+\) T cells indicating none of the TCR are auto-reactive. Taken collectively, the cytokine/chemokine secretion assays and the cytolytic experiments reveal that at intermediate peptide dosage, TCR with \(K_D \sim 1-5 \, \mu M\) are affinity-optimized for CD8\(^+\) T cell activity.

Intracellular Calcium Flux for TCR-transduced CD8\(^+\) T Cells—To assess the effect of TCR binding parameters on an early-stage CD8\(^+\) T cell response we measured intracellular Ca\(^{2+}\) flux (this event occurs within milliseconds of TCR/pMHC contact) (38, 39) upon stimulation with C1R/A2 cells pulsed with low (0.001 & 0.01 \, \mu M), intermediate (0.1 & 1 \, \mu M) and high (5 \, \mu M) concentrations of peptide (Fig. 4). As assessed by changes in Indo 1-AM fluorescence, mean Ca\(^{2+}\) flux gradually increased with the peptide dosage. For CD8\(^+\) T cells expressing the WT TCR, modest flux (about 30% of maximum) was observed upon incubation with C1R/A2 cells pulsed with 0.1 \, \mu M NY-ESO\(_{157-165}\) Peptide (Fig. 4, A and C). For TCR variants G50A, A97L, DM-\(\beta\), and TM-\(\beta\), Ca\(^{2+}\) flux was markedly increased and, unlike for the WT TCR, detectable upon pulsing with 0.01 \, \mu M peptide. However, in the case of the supraphysiologic affinity TCR variants, TM-\(\alpha\), QM-\(\alpha\), and wtc51m, Ca\(^{2+}\) flux was attenuated at this peptide dosage. These results are in accordance with cytokine/chemokine secretion assays and cytolytic experiments. Similar results were obtained for Ca\(^{2+}\) flux experiments using T2 cells pulsed with NY-ESO\(_{157-165}\) peptide (data not shown).

Next we assessed the impact of CD8 co-receptor engagement, an event that occurs within 1 s of TCR/pMHC contact and helps to physically stabilize the interaction (39), on Ca\(^{2+}\) flux. To do this, experiments were conducted using C1R/A2227–228 cells (Fig. 4, B and D). Overall Ca\(^{2+}\) flux was lower as compared with experiments with C1R cells, and differences between the TCR considerably larger. For example, for CD8\(^+\) T cells expressing the low affinity TCR V49I, no Ca\(^{2+}\) flux was observed at any peptide concentration, and for the WT TCR there was little Ca\(^{2+}\) flux even at the highest dosage. For TCR variants G50A, A97L, DM-\(\beta\) and TM-\(\beta\) the responses were substantially higher than WT. Similar to measurements of CD107a up-regulation in response to peptide-pulsed C1R/A2227–228 cells, in the absence of CD8 co-engagement Ca\(^{2+}\) flux is attenuated for T cells expressing supraphysiologic affinity TCR (\(K_D < 1 \, \mu M\)).

Evaluation of TCR-transduced CD8\(^+\) T Cell Activity Levels at Elevated Peptide Concentration—We have evaluated CD8\(^+\) T cell activity levels in response to different APCs pulsed with NY-ESO\(_{157-165}\) Peptide. At intermediate peptide dosage (0.1 to 1 \, \mu M peptide) we measured maximum activity for TCR with \(K_D \sim 1-5 \, \mu M\), the upper limit of natural affinity. Outside of this affinity-optimized range activities were uniformly attenuated.
both in the presence and absence of CD8 co-engagement. Remarkably, comparison of function at elevated peptide concentration revealed that activity was gradually restored for CD8\(^+/\)H11001 T cells expressing supraphysiologic affinity TCR, \(K_D\)\(/H11011/H11021\). For cytokine production this was best demonstrated by the fold-increase in IFN-\(\gamma\) secretion at elevated peptide dosage (Fig. 5, A–C). For cytotoxic assays using T2 cells (A2\(^+/\)H11001/TAP\(^+/\)/H11002) pulsed with graded concentrations of peptide, functional read-out reported as 1/EC\(_{50}\) values (EC\(_{50}\) concentration of ligand giving 50% of maximum lysis) for the entire panel of TCR plotted in order of increasing affinity. F, percentage maximum lysis, \(E_{\text{max}}\), for the different TCR transduced CD8\(^+/\) T cells. G, peak Ca\(^{2+}\) flux values for TCR-transduced CD8\(^+/\) T cells following stimulation with 0.1 \(\mu\)M, H, 1 \(\mu\)M, and I, 5 \(\mu\)M NY-ESO\(_{157-165}\) peptide-pulsed C1R/A2 cells.

DISCUSSION

Using an *in silico* modeling approach we rationally designed a panel of HLA-A\(^*\)0201 restricted NY-ESO\(_{157-165}\)-specific TCR of increasing affinity, and assessed their effect on different CD8\(^+/\) T cell activities. A major finding in this study is that at intermediate peptide dosage of APCs, all T cell responses, including cytokine/chemokine secretion, cytotoxicity and Ca\(^{2+}\) flux, increased with TCR affinity, reaching a plateau at \(K_D \sim 1–5\) \(\mu\)M. Just beyond this threshold, in the low supraphysiologic affinity range, activity levels began to gradually decrease, with major blunting of responses by an extreme supraphysiologic affinity TCR. Thus, the maximum T cell response occurs at intermediate binding parameters.

Previously, based on multimer binding analyses of T cell hybridomas in conjunction with measurement of serine esterase and IL-2 release upon stimulation, Kalergis *et al.* (11) were the first to demonstrate an optimum dwell-time for efficient T cell activation. Here, in addition to determining multimer dissociation half-lives, we measured monomeric TCR/pMHC reaction rates by SPR. Two pairs of TCR among our panel,
DM-β/α and β/β (Fig. 1A), were of particular interest because they each shared near identical half-lives (i.e. differences in affinity resulted from differences in \(k_{\text{on}}\)). Moreover, TCR DM-β and TM-β fell into the upper limit of natural TCR affinity, while TCR TM-α and QM-α, with faster association rates, fell into the low supraphysiologic range.

According to the productive hit rate model of T cell activation it is held that TCR having the same half-life (\(t_{1/2} = \ln 2/k_{\text{on}}\)) will confer equal levels of cellular activity. Thus, we were initially perplexed by the observation that TCR TM-α and QM-α attenuated responsiveness relative to TCR DM-β and TM-β, respectively. Recent studies (40, 41), however, have suggested that for TCR having faster \(k_{\text{on}}\) there can be rapid re-binding of a TCR to the same pMHC complex after dissociation (rather than lateral diffusion of the TCR in the T cell membrane) for a longer pMHC confinement time. Thus, the faster association rates of TCR TM-α and QM-α may result in longer effective half-lives that hinders serial triggering and thereby attenuates T cell activation. This phenomenon of rapid re-binding may explain our observation that the multitimer dissociation half-lives for our TCR variants corresponded to \(K_D\) rather than to \(k_{\text{off}}\) of soluble monomeric TCR as measured by SPR (Fig. 1F).

Another important finding in our study is that increasing peptide dosage led to the restoration of biological activity levels for CD8⁺ T cells expressing TCR of supraphysiologic affinity (\(K_D < \sim 1 \mu M\)), but not for CD8⁺ T cells expressing a subphysiologic one (summarized in Fig. 5). Our observations are corroborated by mathematical modeling (42, 43) demonstrating that, in accordance with the productive hit rate model, as the density of cognate pMHC increases on the surface of an APC, serial triggering becomes less of a determinant of T cell activation for TCR having longer dwell-times. Further, no amount of cognate pMHC can compensate for the reduced T cell responses in the case of TCR having larger \(k_{\text{on}}\) values (short half-life). Indeed, under no conditions were CD8⁺ T cells expressing the low-binding TCR V491 able to achieve maximum activity levels. The affinity model, whereas, predicts that a greater concentration of antigen is always able to compensate for a larger \(k_{\text{off}}\) (43).

Although both of these modeling studies confirmed their mathematical predictions through cytokine measurements, IL-2 responses for hybridoma cells expressing different TCR (42), and IFN-γ for a CD8⁺ T cell clone in response to a panel of plate-captured pMHC variants (mutations were in the MHC molecule itself) (43), here we do so for TCR-transduced CD8⁺ T cells which have greater peptide sensitivity than hybridoma cells (i.e. hybridoma cell function may be impaired) (10). Moreover, we have used a physiological stimulation system (a range of different APCs), which is important because the format of antigen presentation has also been shown to impact T cell responsiveness (44). Further, we have confirmed optimal responses using multiple T cell readouts including cytokine/chemokine secretion, cytotoxicity and Ca²⁺ flux. Interestingly, unlike previous studies demonstrating that the activity of supraphysiologic TCR can be restored through mutation of either the peptide or MHC leading to decreased TCR/pMHC affinity (11, 45), we observed that inhibiting CD8 co-engagement did not restore function for CD8⁺ T cells expressing supraphysiologic affinity TCR.

Another interesting observation in this study was that CD8⁺ T cells expressing supraphysiologic affinity TCR were better able to kill the melanoma cell line Me290, than Me275 (relative to WT TCR, marked with a dashed line in Fig. 3, A and B). Although we have been unable to demonstrate it experimentally, we hypothesize that Me290 cells express higher levels of cognate pMHC and thus CD8⁺ T cells expressing the higher affinity TCR are less dependent on serial triggering in their killing of these cells. These observations have important implications for the interpretation of functional read-outs for T cell clones derived from patients under clinical study; lower cellular activity does not necessarily mean that the TCR are of weak affinity as the TCR may in fact bind the ligand too strongly for efficient serial engagement.

Our finding that activity levels are restored for CD8⁺ T cells expressing higher affinity TCR at higher peptide dosage may also have implications in clonal selection during the primary immune response. Relative high density of a peptide of viral origin at the start of an infection, for example, may select for higher affinity/avidity TCR (46) that enable accelerated T cell responses (45). For a tumor-associated peptide that may be present at lower density, whereas, clones expressing lower affinity/avidity TCR more efficient in serial triggering may be preferentially selected.

As described, TCR/pMHC interactions can be modified to alter cellular function by changes in sequence of the peptide (i.e. APL), the MHC molecule, or the TCR (11). Many studies assessing the impact of TCR/pMHC binding parameters on T cell activity have used APLs but a caveat is that modifying the peptide sequence may affect not only binding by the TCR, but also its stability within the MHC complex itself (7). This may in part explain the observation that APLs can differ not only in their potency but may also cause heteroclitic T cell responses (3). Moreover, although APLs have been shown to more readily induce T cell responses, it has been demonstrated that T cell responses in cancer patients to unmodified peptide have stronger tumor reactivity than those induced by APL (47). Here our TCR variants yielded CD8⁺ T cell activity profiles that differed quantitatively but not qualitatively from one another.

Although our panel of TCR have not been assessed in a mouse model (ongoing study), others have shown that CD4⁺ T cell activation is attenuated in vivo in response to high-affinity peptide ligand (48), and that optimal activity occurs in response to ligands with intermediate TCR/pMHC half-lives (49). Furthermore, it has been demonstrated that vaccination with peptide ligands of intermediate affinity elicit more potent tumor reactive CD8⁺ T cells in vivo and best tumor control (50). Therefore, we predict that our TCR variants falling within the upper natural limit of natural affinity \((K_D \sim 1–5 \mu M)\) will likewise confer maximum activity in vivo, and that the supraphysiologic affinity TCR will blunt responses.

An important open question in the field of T cell activation is if TCR having different gene-usage but sharing specificity for A2/NV-157–165 or TCR specific for other pMHC complexes, will confer optimal function within these same binding parameters. It is possible that greater differences in conformational change (heat capacity) of a TCR upon pMHC engagement may shift the range (51). Further, although data
presented here support the productive hit rate model for T cell activation, how TCR/pMHC binding mediates differences in TCR triggering, the transduction of signal across the plasma membrane, has yet to be fully elucidated (reviewed by Ref. 52).

There is increasing evidence that TCR/pMHC contact yields conformational change to TCR constant regions, which in turn effects TCR clustering and CD3 complex orientation etc (53, 54). In silico modeling provides an important tool in the characterization of protein/protein interactions and should thus be helpful in addressing many of these important questions. Importantly, our work demonstrates that a rational design approach can be used with remarkable precision in the fine-tuning of TCR/pMHC binding for achieving maximum CD8+ T cell activity levels.

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