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Supplemental Information

T Cell Receptor Signaling Is Limited by Docking Geometry to Peptide-Major Histocompatibility Complex

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Supplemental Experimental Methods

Cloning

Primary 42F3 T cells were mixed with 1mL TRIzol Reagent (Invitrogen) on ice then incubated for 5 minutes at room temperature. 200μl chloroform was added and mixed by shaking for 15 seconds followed by room temperature incubation for 2-3 minutes. Treated cells were centrifuged at 12000*g* for 15 minutes at 4°C. Aqueous RNA phase was transferred to a fresh tube and 500 μl isopropyl alcohol/1□L TRIzol was added and mixture was incubated for 10 minutes at room temperature. Sample was centrifuged for 10 minutes at 12000*g* at 4°C. 1mL 75% ethanol/1mL TRIzol was mixed with the precipitant and centrifuged for 5 minutes at 7500*g* at 4°C. Supernatant was removed and the pellet was allowed to air dry for 5-10 minutes. RNA was resuspended in 50μl RNAse-free water and incubated for 10 minutes at 60°C. RNA concentration was quantified by analyzing by A_{260}/A_{280}. Total RNA was converted to cDNA using a SuperScript First-Strand Synthesis System (Invitrogen) creating heterogeneous cDNA. 42F3 was cloned from bulk cDNA by nested PCR as previously described (Baker et al., 2002) and cloned using a TOPO TA cloning kit (Invitrogen) using Vα fwd 5' gctcagtcagtgacacagccc 3' and Vβ fwd 5' gaggctgcagtcacccaaagc 3' primers.

The V regions of the α and β chain were spliced by overlapping extension to form a Vα-$(Gly_4Ser)_4$-Vβ scFv. The scFv fragments were cloned in frame with a C-terminal 6xHis-tag into pET22b to allow for periplasmic secretion in BL21-Codon Plus E. coli (Stratagene). Two mutations in the Vα3.3 (W82R and L44P) and five mutations in the Vβ8.3 (G17E, G42E, H47Y, L175T and L177S) were made using the QuickChange Site-Directed Mutagenesis method (Stratagene) to create the 42F3 Mut7 scFv TCR as previously described for 2C (Colf et al., 2007).

The wt Vα3.3 and Vβ8.3 regions of the 43F3 TCR were also fused in frame with human constant domains containing engineered stabilizing cysteines to form the 42F3 V_mC_h TCR (Boulter et al., 2003). 42F3 V_mC_h was ligated into pAcGP67a vector encoding a C-terminal acidic GCN4 zipper-BAP-6xHis tag (α) or a C-terminal basic GCN4-zipper-6xHis tag (β). The 42F3 V_mC_h regions were also cloned into

pET28a for expression as inclusion bodies as previously described (Boulter et al., 2003).

42F3 T cell lines

The 42F3 alpha and beta V genes were synthesized in frame with the mouse TCR alpha and beta constant regions (Genscript) and cloned into retroviral transduction vector MSCV. Constructs were verified by sequencing. 30μg 42F3-MSCV DNA and 60μg Lipofectamine 2000 (Invitrogen) were incubated at room temperature in 3ml Opti-mem media (Gibco #11058) for 20 minutes. After removing DMEM culture supernatant, DNA/lipofectamine mixture were incubated with $3x10^6$ adherent Plat-E cells for 3 hrs at 37°C. DNA/lipofectamine supernatant was removed and Plat-E cells were recovered overnight at 37°C in a poly-L-lysine coated 100mm tissue culture dish. DMEM media was removed and Plat-E cells were incubated in RPMI 1640 at 37°C overnight. 58-/- cell lines with and without CD8 (Holler et al., 2003) were transduced by centrifuging $2x10^6$ cells in a 24-well plate (Corning costar) with 1.5ml of filtered culture supernatant from transfected Plat-E cells for 45 minutes at 652*g*. Cells were incubated with Plat-E supernatant for 4hrs at 37°C prior to incubation with additional 1.5mL of filtered Plat-E supernatant. 1mL supernatant was removed, and cells were cultured overnight at 37°C. Transduced cells were transferred to T25 flask with 5mL RPMI 1640 and cultured at 37°C and 5% CO_2. The brightest 1% of Vβ positive cells were sorted by staining with 10μg/ml biotinylated anti-Cβ antibody followed by 1.7μg/mL SA-PE. 42F3 cells were cultured in RPMI 1640 with 10% FCS and 0.5mg/mL G418.

Protein expression and purification

For the single-chain 42F3 scFv used in the QL9 complex (Figure 1), BL21 *E. coli* were transformed with 42F3 "Mut7." Single colonies grown in 50ml overnight cultures were used to inoculate 1L cultures of LB broth at 30°C for 12hrs. Cells cultures were centrifuged at 4500*g* for 15min at and transferred to 1L TB media at 21°C. Cells were allowed to recover for 1hr before induction with IPTG and

incubated at 21°C for 5 hours. The scFv TCR was harvested by a sucrose/edta osmotic shock method (Colf et al., 2007; Jones et al., 2006) and dialyzed against 10mM TRIS pH8 and 200mM NaCl. Protein was purified by Ni-NTA chromatography (Qiagen) followed by Superdex 75 size exclusion chromatography (Amersham). The H2-L^d class I MHC platform (SBM2) was expressed, purified and refolded as previously described (Colf et al., 2007; Jones et al., 2006) with exogenous peptides QL9, p4B10, p5E8 and p3A1 (Genscript).

Biotinylated V_mC_h chimeric TCRs were expressed in Hi5 insect cells by baculovirus coinfection of α chain and β chains in the presence of secreted BirA ligase and 50μM biotin. Cells were pelleted by centrifugation and the cell media was primed for Ni-NTA capture by the addition of 100mM Tris pH8.0, 1mM $NiCl_2$ and 5mM $CaCl_2$. Precipitate from the media was removed by centrifugation and TCRs were captured by batch Ni-NTA purification and further purified by Superdex 200 (Amersham) size exclusion. Biotin incorporation was assayed by band shift using SDS-PAGE in the presence of saturating streptavidin.

For the p3A1 complex ,the 42F3 V_mC_h α, β were co-infected into insect cells in the presence of 4μM kifunesine and cleaved with EndoH to produce partially deglycosylated 42F3 TCR for crystallography. Insect expressed 42F3 TCR used for crystallography and SPR were cleaved with 3C protease to remove zippers and further purified by size exclusion. 42F3 V_mC_h was also produced in *E.coli* by refolding from inclusion bodies by methods previously described (Clements et al., 2002), and purified by ion exchange on DE52 resin followed by 60% ammonium sulfate precipitation and Superdex 200 (Amersham) size exclusion. N-terminal 6xHis-tags were removed by thrombin (GE healthcare) digest overnight at RT.

Yeast displayed L^d-peptide libraries

H2-L^d was displayed as a minimal $\alpha1\alpha2$ variant of H2-L^d called m31, (Jones et al., 2006). M31 was converted into a single chain pMHC with a C-terminal tethered peptide (Aga2-L^d_{W167A}-pep) by incorporating the mutation W167A. The W167A mutation created an opening at the end of the L^d $\alpha2$ helix for the peptide

Gly-Ser linker to pass without influencing the binding of the TCR. Aga2-L^{d}_{W167A}-pep was cloned into the yeast display vector pCT302. A degenerate primer containing the peptide library annealing to the peptide-associated linker nucleotide sequence were used to PCR amplify combinatorial libraries. A second PCR was used to further amplify the total mass of DNA and to extend the 5' and 3' ends of the amplicon with 50bps complementary to the vector sequence. Libraries were then recombined into linearized vector using homologous recombination in yeast (Chao et al., 2006). Briefly, 1mL of electro-competent yeast were prepared as previously described and transformed with 100μg extended library amplicon and 20μg of linearized pCT302. Yeast cultures were allowed to recover in YPD for 1hr and grown in SDCAA media. Cultures were split twice to 10^7 cells/ml in SDCAA and grown for 24hrs shaking at 30°C. Transformants were calculated from the number of colonies formed on plates from a serial dilution of the recovered electroporated yeast cultures. The number of transformed yeast for each library were $1.8x10^8$, $1.0x10^8$ and $5.7x10^7$ for the Random, TCR contact and MHC contact libraries respectively.

42F3-Tetramer selection of yeast clones

Freshly cultured cells from each yeast library were resuspended in SGCAA pH 4.5 at a concentration 10^7 cells/mL and were grown shaking at 20°C for 48 - 72 hours. After induction, the yeast cells were washed once with PBS+0.1%BSA (PBSB) and incubated with 470nM preformed 42F3-tetramers assembled on SA-PE (Invitrogen) and 1:100 dilution of anti-HA-Alexa488 (Invitrogen) for 3hrs at 4°C while slowly tumbling to maintain cells in suspension. After incubation cells were washed twice with PBSB and resuspended in FACS buffer immediately before sorting on a FACS Aria (BD). During the first round of selections the brightest 2.5% of the TCR-Tet+/HA+ population was sorted into a single culture. The sorted cells were grown overnight at 30°C in SDCAA to a cell concentration of $5.0x10^7$-$1.0x10^8$ cells/mL before the yeast were induced again and the process repeated. After the first round of selection, the brightest 1% of TCR-Tet+/HA+ was sorted until greater than 10% of the yeast population could be resolved from the unstained population

by flow cytometry. Each selection oversampled the total number of selected clones from the previous round by at least 10 fold to maintain diversity throughout the enrichment. Enriched populations were serially diluted to individual clones and grown on SDCAA plates. Individual yeast colonies were picked and grown in 1mL SDCAA in a 96 deep well block for 36hrs, shaking at 30°C. Yeast clones were induced by resuspending 10^7 cells in 1mL SGCAA pH4.5 in a 96 deep well block and grown for 48hrs, shaking at 20°C. Yeast cultures were stained with 100nM preformed 42F3-tetramers while rocking on ice for three hours in a 96 well plate. Fluorescence was detected and analyzed using a C6 flow cytometer with an autosampler (Accuri). Data analysis was carried out on Flowjo and CFlow software. Individual plasmids from yeast clones were harvesting using the Zymoprep yeast plasmid mini-prep II kit (Zymo Research) and sequenced (Sequetech).

Surface plasmon resonance

Binding affinities and kinetics of purified 42F3 V_mC_h with the different single-chain pMHC were determined by surface plasmon resonance on a Biacore T-100 (GE). Yeast displayed clones were amplified from pCT302 and cloned into the pETDuet1 (Novagen) bicistronic vector with an N-terminal biotin acceptor peptide (BAP) tag (BAP-L^d_{W167A}-pep) and coexpressed with BirA ligase with the addition of 100μM biotin upon induction. Inclusion bodies were purified and refolded as previously described (Colf et al., 2007; Jones et al., 2006). Refolded biotinylated BAP-L^d_{W167A}-pep was coupled to a SA-chip (GE Healthcare) and association and dissociation was measured at concentrations of 0-100μM 42F3 V_mC_h at 25°C with a flow rate of 50μl/min without regeneration between injections. Resonance curves were fit to a 1:1 Langmuir steady-state model and affinities were calculated for each Bap-L^d_{W167A}-pep measured. Kinetic parameters k_{on} and k_{off} were also calculated for several pMHC however k_{off} values often fell well outside the limits of detection for this instrument (>0.5s^{-1}) or poorly fit the data based on the residual analysis and therefore the 3D kinetics were considered only qualitatively. Kinetics fits for two

pMHC complexes resulted in K_D and R_{max} values that converged with the values derived from steady-state fits and we present them for comparison in **Table S2**.

T cell activation assays

LM1.8-L^{d}W97R cells (1x10^5) and peptides (10μM) were diluted in RPMI and incubated in a 1:1 effector to target ratio with CD8αβ$^+$ or CD8αβ$^-$ 42F3 T cells for 20 hrs at 37°C. Alternatively, recombinant H-2L^d:Ig fusion protein (BD Biosciences) was loaded with exogenous peptides by incubating 100nM H2-L^d:Ig in the presence of 10μM peptide overnight at 37°C. Peptide-loaded H2-L^d:Ig dimers (50μL) were immobilized overnight at 4°C in a 96-well tissue culture plate (Corning Costar)(Chervin et al., 2009). CD8αβ$^-$ 42F3 T cells (10^5) diluted in RPMI were incubated for 20hrs at 37°C in wells with immobilized pep/H2-L^d:Ig. Supernatants were assayed for cytokine release by IL-2 ELISA (BD Pharmingen). Experiments were done in duplicate in two independent experiments.

Biotinylated peptide competition assays

Biotinylated p4B3 (Princeton Biomolecules) peptide was diluted in RPMI at varying concentrations and added to 10^5 APCs for 2hrs at 37°C. Cells were washed twice with PBS+0.5%BSA and incubated with 100nM SA-Alexa647 for 15min at 4°C. Cells were washed twice again with PBS+0.5%BSA and analyzed for fluorescence signal. Competition assays were carried out using APCs preloaded (5min) with 5μM biotinylated p4B3 and then mixed with varying concentrations in triplicate of unlabeled peptide for 2hrs at 37°C.

Tetramer staining

Washed LM1.8-L^{d}W97R APCs (2x10^5) were incubated on ice for 3hrs with 10μM peptide (MCMV, QL9, p3A1, p4A1 or p5E8) and 470nM preformed 42F3-tetamers diluted in PBSB. After incubation, cells were washed twice with cold PBSB to remove unbound tetramer. Cells were analyzed for PE fluorescence on a C6 flow cytometer (Accuri).

Likewise, washed 42F3 T cells ($1x10^6$) were incubated on ice for 3hrs with 470nM preformed single-chain pMHC-tetramers encoding QL9, p3A1, p4B10 and p5E8 diluted in PBSB. After incubation, cells were washed twice with cold PBSB to remove unbound tetramer. Cells were analyzed for PE fluorescence on a C6 flow cytometer (Accuri).

Determining molecular site density

Site densities of TCR on hybridoma cells and pMHC on RBC or bead surfaces were measured with flow cytometry. The 42F3 hybridoma cells were stained with PE-conjugated anti-mouse Vβ8 (F23.1, BD bioscience). The pMHCs densities were estimated by the available biotin binding sites on the RBC or bead surface, which were measured from the same batch RBCs or beads coated with biotin-tagged H2-K^b-OVA (instead of H2-L^d) stained by PE-conjugated anti-mouse H2-K^b-OVA (eBioscience, Clone 25-D1.16). The total number of molecules and thus site density (total molecule number divided by cell surface area) was quantified using PE standard beads (BD Quantibrite PE Beads, BD) as a reference.

Micropipette adhesion frequency assay

The 2D effective affinity was measured with the micropipette adhesion frequency assay (Chesla et al., 1998). Briefly, a pMHC-coated RBC and a 42F3 CD8- T cell were gently aspirated by two opposing micropipettes in L15 media supplemented with 5 mM HEPES/1% BSA. The RBC was controlled by a piezoelectric translator to gently contact the 42F3 T cell for a certain time (t_c) and then retracted. During retraction, adhesion (if occurred) was visually identified by the stretch of the RBC membrane. Adhesion frequency (P_a) is defined by the number of adhesions divided by the total contact number touches (50 for each cell pair). The effective affinity was calculated by:

(1) $A_c K_a = -\ln (1-P_a)/(m_r m_l)$

Where A_c, K_a, m_r, and m_l represent contact area, 2D affinity, TCR site density, and

pMHC site density, respectively.

Thermal fluctuation assay

The 2D off-rate of TCR/pMHC dissociation was measured with the thermal fluctuation assay on a customized bio-membrane force probe (BFP) (Chen et al., 2008). BFP is a high-tech version of the micropipette system with a super-sensitive force probe, assembled by attaching a streptavidinylated bead coated with biotinylated pMHC to the apex of a biotinylated RBC that was aspirated by a micropipette. The well-defined optical image of the bead allows customized image analysis software to track the RBC deflection with a ± 3nm (std) spatial and 0.7 ms temporal resolution. A 42F3 T cell aspirated by an apposing micropipette was driven by piezoelectric translator to gently contact the pMHC-coated bead, retract to a null position where the compressive force just vanished, and hold for a certain period to allow bond association and dissociation under zero force. Bond association/dissociation was indicated by sudden changes in the thermal fluctuations of the bead. Lifetime was counted from the instant when the amplitude of the thermal fluctuation dropped to the instant when it rose to baseline level. Hundreds of lifetimes were measured for each peptide and their distributions were analyzed in a semi-log plot. The negative slope equals the off-rate.

Crystallization

42F3 Mut7 scFv and QL9/SBM2 were mixed together and concentrated in a 1:1 molar ratio with a final concentration of 350μM. De novo crystallization was carried out in a sitting drop plate using 7% (w/v) PEG 17,500, 100mM Bicine pH 8.25 as the well solution and mixing 1:1uL drops. Crystals were stepwise soaked into well solution with 30% (v/v) glycerol and flash frozen in liquid nitrogen.

Endo-H treated 42F3 V_mC_h was digested with 3C protease to remove affinity tags and co-purified with p3A1/SBM2 by size exclusion. The complex was concentrated to 150μM complex and set up in hanging drop plates for crystallization. Crystals formed de novo by mixing 20% PEG 3350, 10% sodium

tacsimate pH 6.0 (Hampton) in a 1:1 (v/v) drop with the TCR-pMHC complex. Crystals were soaked into well solution with 15% ethylene glycol and flash frozen in liquid nitrogen.

Refolded 42F3 V_mC_h was co-purified with p4B10/SBM2 and the complex was concentrated to 150μM. Crystals formed de novo by mixing a 16% PEG 3350, 0.2M ammonium nitrate and 0.1M Bis-Tris pH 6.6 in a 1:1 (v/v) drop with the TCR-pMHC complex. Crystals were soaked stepwise into well solution with 20% ethylene glycol and flash frozen in liquid nitrogen.

Refolded 42F3 V_mC_h and p5E8/SBM2 were purified by size exclusion and mixed together and concentrated in a 1:1 molar ratio with a final concentration of 150μM. Crystals formed de novo by mixing 1.1M lithium sulfate, 1% (v/v) PEG 200 and 0.1M sodium acetate pH 4.4 in a 1:1 (v/v) drop with the TCR-pMHC complex. Crystals were soaked stepwise into well solution with 35% ethylene glycol and flash frozen in liquid nitrogen.

Diffraction data were collected at the Stanford Synchrotron Radiation Laboratory (Menlo Park, California, USA) on beamlines 9-2, 11-1 and 12-2. The diffraction data was indexed and scaled using either HKL2000 (Otwinowski et al., 1997) or MOSFLM/SCALA (Leslie, 1992). The structures were solved by molecular replacement using the program PHASER (McCoy, 2007). Refinement was carried out using REFMAC (Murshudov et al., 1997), CNS (Brunger et al., 1998), or PHENIX (Afonine et al., 2010) using NCS restraints when applicable. Structural models were manipulated in COOT (Emsley and Cowtan, 2004). Figures were made in PYMOL (DeLano, 2002).

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	42F3 Mut7 scFv SBM2/QL9	42F3 V_mC_h EndoH SBM2/p3A1	42F3 V_mC_h SBM2/p4B10	42F3 V_mC_h SBM2/p5E8
Data collection				
Space group	$P2_12_12_1$	$P2_12_12$	$P6_122$	$R32$
Cell dimensions				
a, *b*, *c* (Å)	102.49, 109.79, 213.59	197.19, 61.67, 69.82	102.09 102.9 323.08	291.28, 291.28 291.25
a, b, g (°)	90, 90, 90	90, 90, 90	90, 90, 120	90, 90, 120
Resolution (Å)	50-2.75 (2.85-2.75)	58.9-2.12 (2.23-2.12)	80.84-2.75 (2.9-2.75)	50.0-3.1(3.13-3.10)
R_{merge}	0.049 (0.755)	0.069 (0.379)	0.088 (0.810)	0.126 (0.805)
I / sI	36.7 (2.1)	13.1 (3.8)	23.7 (4.0)	14.9 (1.5)
Completeness (%)	97.1 (89.3)	99.8 (99.5)	100 (100)	99.7 (97.1)
Redundancy	7.0 (5.9)	4.3 (4.4)	19.2 (18.2)	6.2 (4.6)
Refinement				
Resolution (Å)	37.45-2.75	29.70-2.10	50.42-2.75	45.31-3.10
No. reflections	58631	49112	26761	85205
R_{work} / R_{free} (%)	23.7/26.1	20.6/23.7	22.7/29.1	22.6/25.6
R.m.s. deviations				
Bond lengths (Å)	0.006	0.003	0.009	0.021
Bond angles (°)	0.969	0.670	1.2	1.73
Ramachandran Statistics				
Favored Region	95.4	95.6	92.2	93.7
Allowed Region	4.0	4.2	7.6	6.2
Outlier Region	0.6	0.2	0.2	0.1

nomenclature key:
•SBM2-peptide – L^d-α1/α2 platform refolded with respective peptides.
•42F3 Mut7 scFv – single-chain Fv of 42F3.
•42F3 V_mC_h EndoH – full-length 42F3 αβ ectodomain heterodimer produced in baculovirus in the presence of kifunensine and endo-H treated.
•42F3 V_mC_h - full-length 42F3 αβ ectodomain heterodimer produced by refolding from E. coli.

Table S1 (related to Figures 1, 5, and 6) - Crystallographic Statistics

	pMHC	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (μM)	R_{max} (RU)	$t_{1/2}$ (s)
Kinetic	3A1	$2.01x10^5$	0.752*	3.7	239.2	0.92
Steady-state	3A1	-	-	3.9	197.5	-
Kinetic	5F1	$2.08x10^5$	1.690*	8.1	174.9	0.41
Steady-state	5F1	-	-	5.1	154.8	-

*Parameter falls outside the detection range of the Biacore T100

Table S2 (related to Figures 2 and 3) - Kinetic analysis of 42F3 binding to p3A1 and 5F1 pMHC

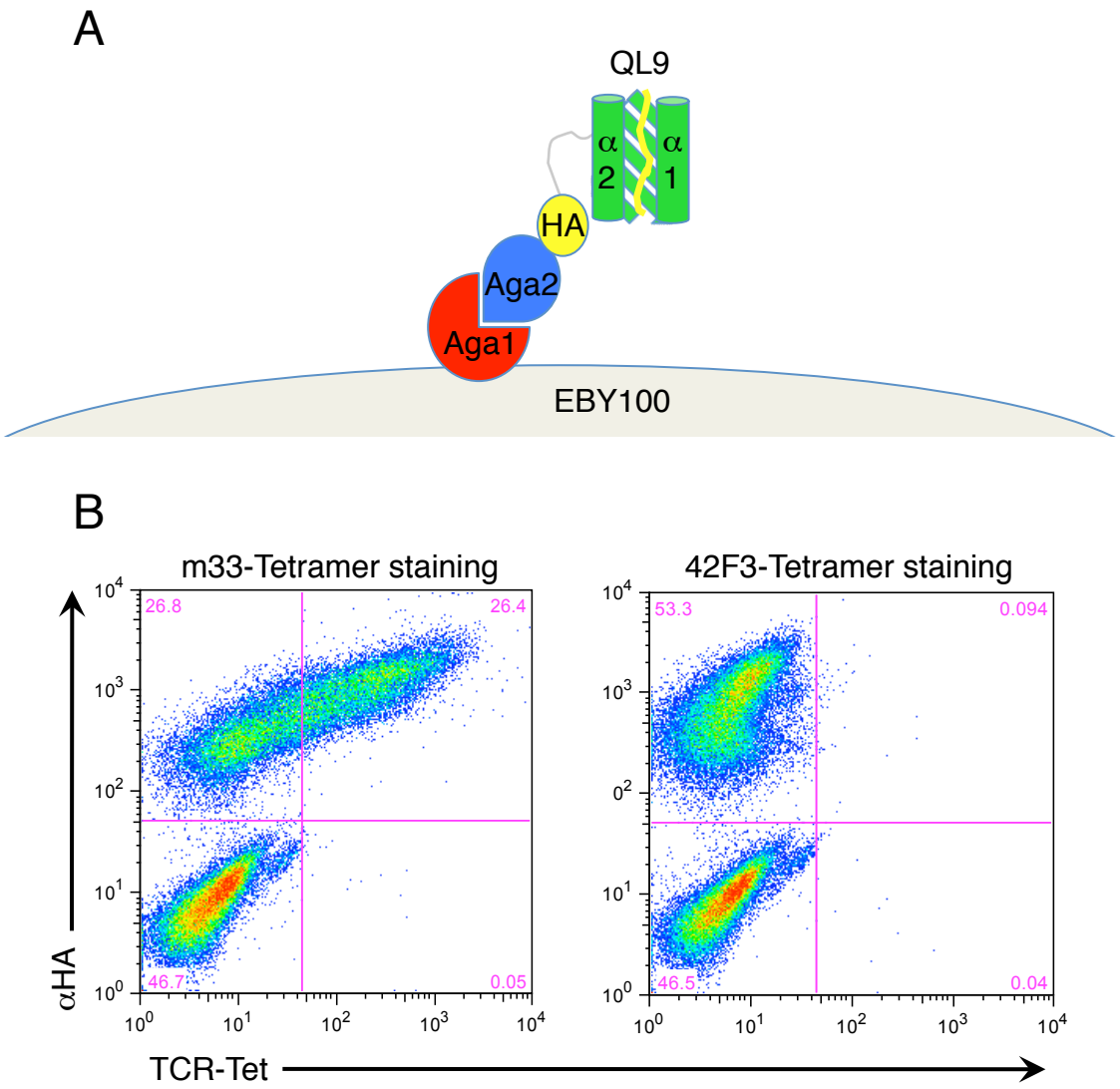

Figure S1. Yeast display of single chain H2-L^{d}-QL9 (related to Figure 2). A cartoon schematic of a yeast displayed single chain 'mini' H2-L^{d} tethered to QL9. (**B**) TCR-tetramer staining of yeast, displaying the Aga2-L$^{d}_{W167A}$-QL9 with QL9/H2-L^{d} specific TCR tetramers. The high affinity TCR m33 robustly stains the yeast, while 42F3 staining is only weakly detectable, in accord with their K_D's.

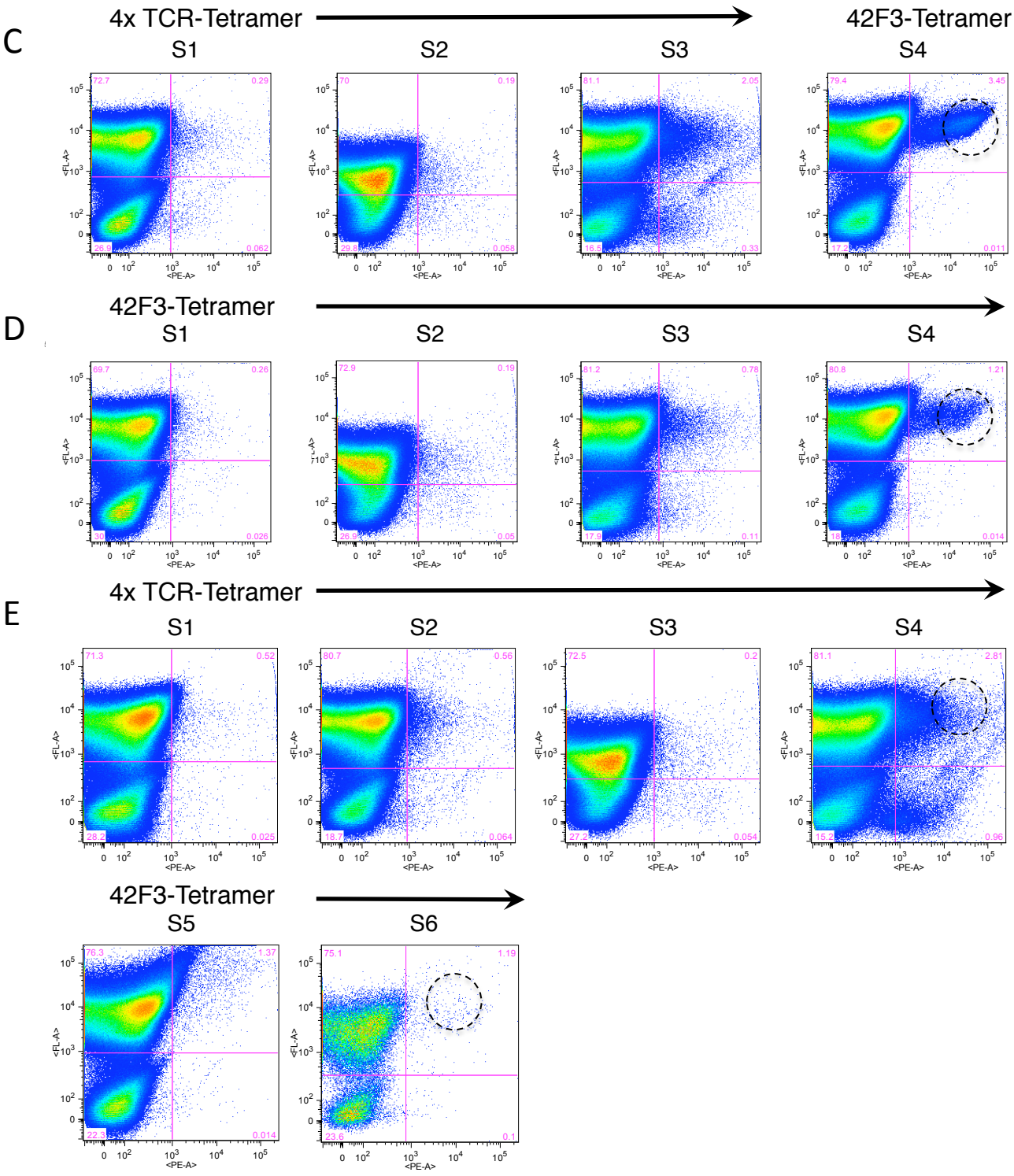

Figure S1 (cont'd). Yeast displayed H-2L^{d}-peptide library selections (related to Figure 2)**.**
42F3-tetramer stains during rounds of enrichments of yeast populations expressing (**C**) the TCR contact library, (**D**) the MHC contact library or (**E**) the random library tethered to the Aga2-L$^{d}_{W167A}$ variant. Rounds of selections were carried out either by FACS or MACS and both surface display and TCR binding were detected in the enriched populations. HA staining is detected along the y-axis, while TCR tetramer staining is detected along the x-axis.

F

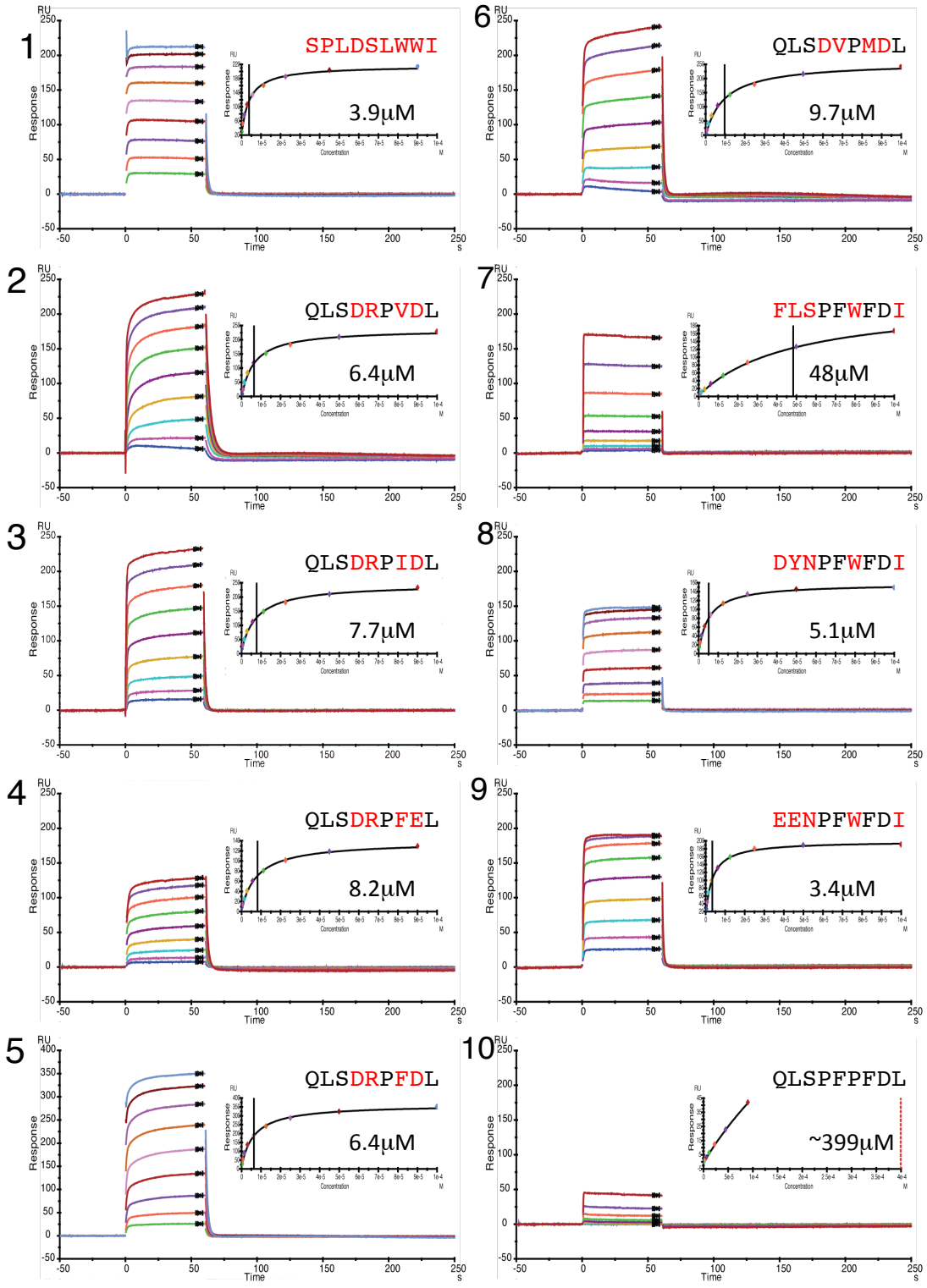

Figure S1 (cont'd). 3D binding affinities of 42F3 for selected peptide-MHC measured by surface plasmon resonance (related to Figures 2 and 3).
F) Affinities of 42F3 for refolded sc BAP-H2-L^{d}_{W176A}-pep constructs encoding (**1**) p3A1, (**2**) p4A1, (**3**) p4A2, (**4**) p4A3, (**5**) p4A4, (**6**) p4B10, (**7**) p5E8, (**8**) p5F1, (**9**) p5F2, and (**10**) QL9 as determined by SPR. Inset are the peptide sequence, steady-state fits and affinity of each interaction assuming a 1:1 Langmuir model. The pMHC was biotinylated through a C-terminal BAP tag for immobilization on an SA-chip.

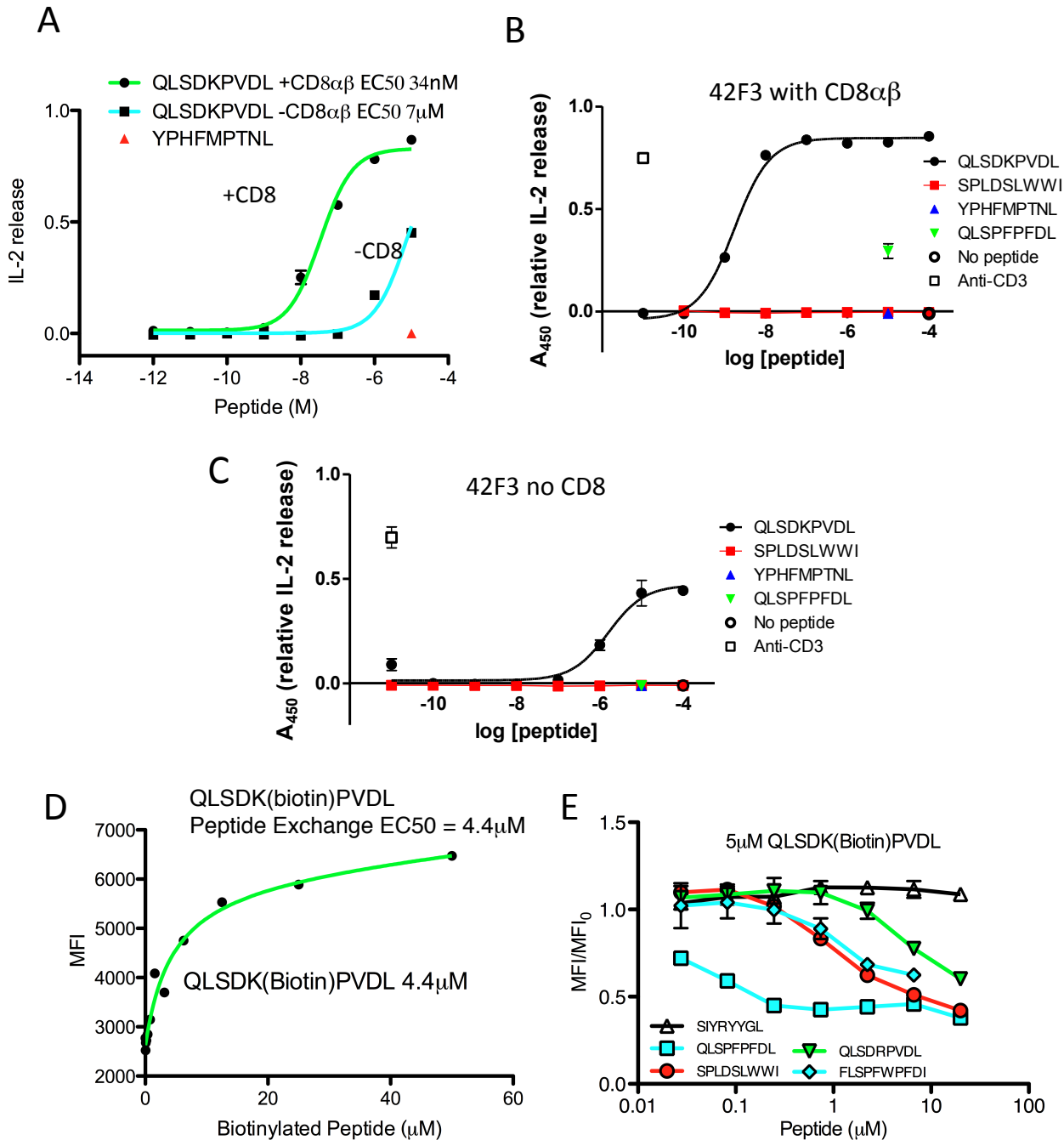

Figure S2. Agonist and non-stimulatory peptide MHC loading and activity (related to Figure 3).
(**A**) $CD8^+$ and $CD8^-$ 42F3 T cell activation over a range of p4B3 agonist concentrations on APCs. (**B**) Dose response curves for agonists and p3A1 non-stimulatory peptides up to a [peptide] of 100 micromolar for 42F3 plus CD8 cells and **(C)** minus CD8 cells. **(D)** Biotinylated p4B3 titration on APCs demonstrates loading into H-$2L^d$. Peptide exchange was assayed by SA-PE binding to the APC. (**E**) Competitive inhibition of 5uM p4B3 binding in the presence of agonist peptides (cyan - CD8 dependent, green - CD8 independent) and the non-agonist p3A1 (red) over a range of concentrations. The error bars indicate ± standard error of the mean.

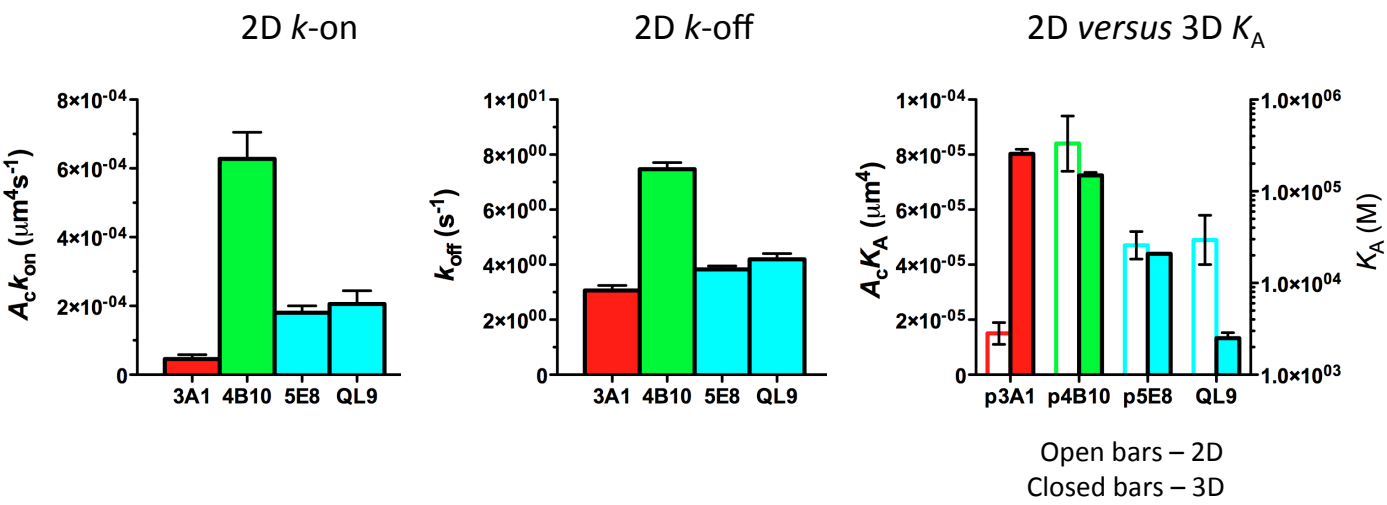

Figure S3. 2D kinetics derived from *in situ* measurements on cells (related to Figure 4). 2D $A_c k_{on}$ (left) and 2D k_{off} (middle) for biotinylated sc H2-L^d_{W167A}-peptide constructs encoding p3A1, p5E8, p4B10 and QL9 on $CD8^-$ 42F3 T cells. The 2D affinities ($A_c K_A$) (outlined bars) are compared to SPR-derived 3D affinities (K_A) (filled bars) of these interactions (right). The error bars indicate ± standard error of the mean.

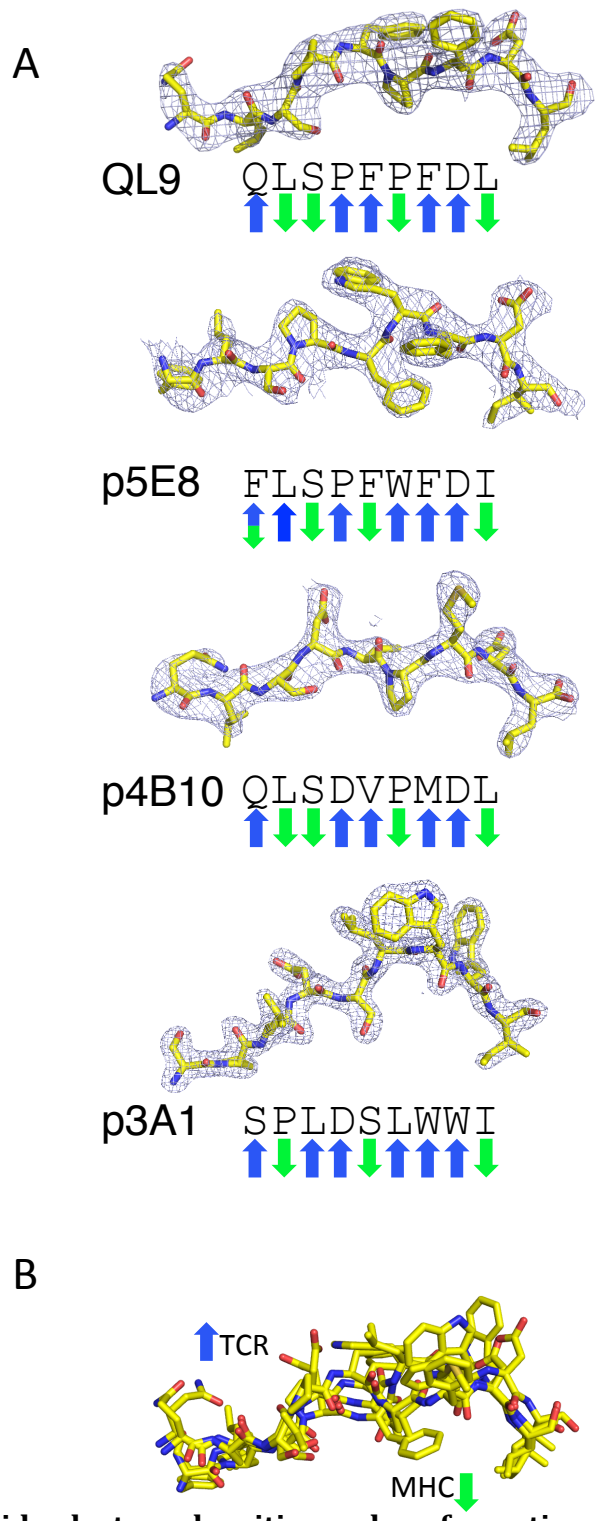

Figure S4. Peptide electron densities and conformations in the structures (related to Figure 5)**.** (**A**) Electron density (grey) of QL9, p3A1 p4B10 and p5E8 peptides contoured to 1.5σ of the $2F_o$-F_c maps. (**B**) Overlaid peptides. All peptides are depicted as sticks (yellow). Peptide positions poised for TCR engagement (blue arrow) and buried in the MHC (green arrow) are indicated.

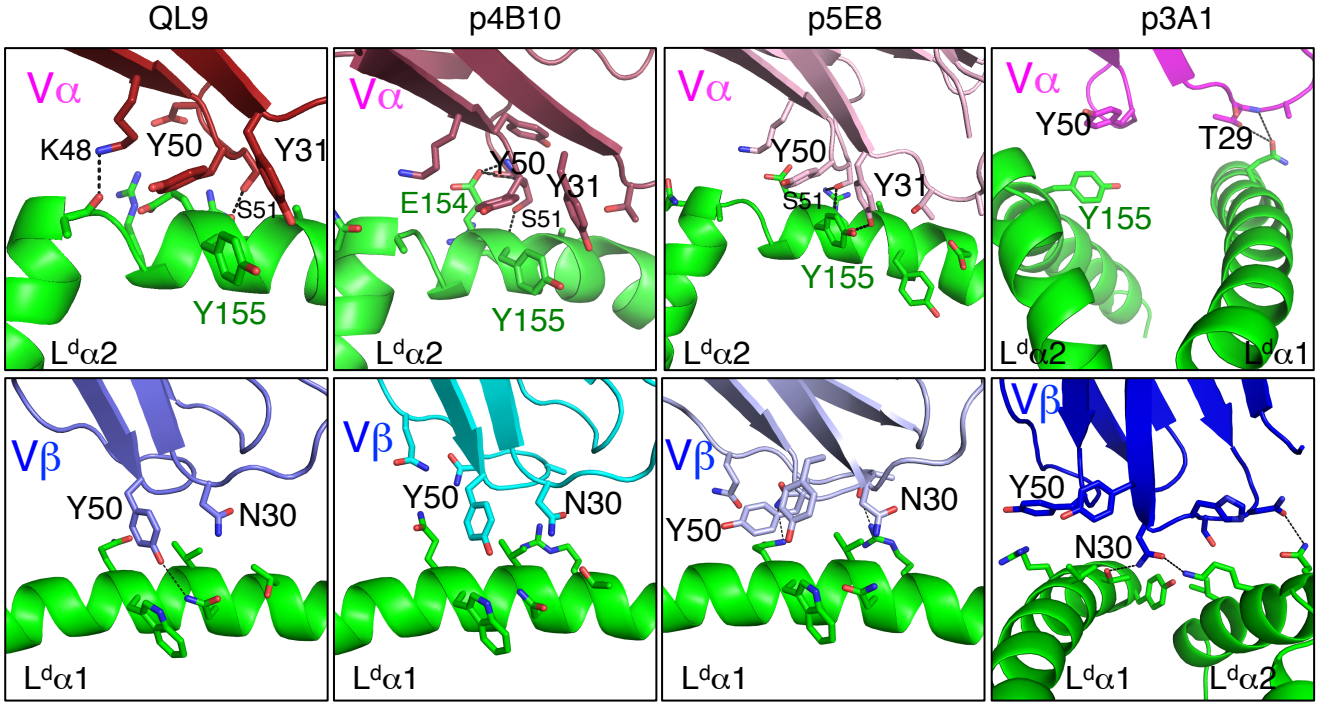

Figure S5. Germline-mediated contacts between 42F3 V-regions and H2-L^d (related to Figures 6 and 7). Contacts made by the Vα (top) and Vβ (bottom) germline CDR1-2 loops to the H2-L^d surface. Residues making van der Waals contacts are depicted as sticks, hydrogen bonds are depicted with dashed lines. The MHC is colored green. 42F3α is colored firebrick (QL9), lightpink (p5E8), raspberry (p4B10) and magenta (p3A1). The 42F3β is colored slate (QL9), lightblue (p5E8), cyan (p4B10) and blue (p3A1).