Supplementary Information

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Supplementary Table 1. Summary of high-affinity phage clones isolated following 3 rounds of selection. Approximately 12 random clones giving positive ELISA scores were sequenced from both the CDR2 and CDR3 pooled experiments. Only CDR2β and CDR3α mutations were present.

CDR3α	Abundance in output	CDR2β	Abundance in output
CAVRTNSGYALNFG	**0**	**YYEEEE**	**0**
CAVRSAH**GYALNFG**	**2**	**Y**VRG**EE**	**3**
CAVRSAHD**YYLNFG**	**1**	**Y**VRGV**E**	**2**
CAVRGAHD**YALNFG**	**6**	**Y**VRGQ**E**	**1**
CAVRGAHD**YSLNFG**	**1**	**Y**VRGM**E**	**1**
CAVRGHNDYF**LNFG**	**1**	**Y**AFGT**E**	**1**
		YALG**EE**	**2**
		YAFG**EE**	**1**
		YSFG**EE**	**1**

RESULTS: Following 3 rounds of selection, phage-ELISA screening of randomly selected clones produced a high frequency (>90%) of binders in the CDR2 output (see supplementary methods below). Sequence analysis revealed a highly convergent set of mutants derived exclusively from the CDR2β library (Supplementary Table 1). Of particular note is the selection of an arginine residue (seen in the VRG motif) that represents a dramatic charge reversal over the glutamate string of the germline-encoded sequence. However, related mutations containing bulky hydrophobic residues at this position were also abundant in the output (Supplementary Table 1), and were shown by SPR to exhibit comparable binding kinetics (data not shown) with both Arg and Leu mutants yielding a >14-fold improvement in the dissociation constant over the parental clone. Thus, the higher abundance of Arg in the output may simply reflect better propagation and/or display characteristics of this sequence. CDR3 sequences also appeared highly convergent with a mutant population derived exclusively from the CDR3α library (Supplementary Table 1). The failure to recover either CDR2α or CDR3β mutations from this pooled library experiment may indicate that these loops might either make no contacts, or alternatively already make near optimal contacts with the HLA A2-SL9 antigen.

Supplementary Table 2. BIAcore™ surface plasmon resonance (SPR) analysis of HIV 868 TCR binding to 8 different biotin-tagged HLA A2-SL9 antigenic variants including SLYNTVATL (index (SL9)), immobilized to a streptavidin-coated flow cell. The other SL9 antigenic variants used were : SL**F**NTVATL (3F), SLYNT**I**ATL (6I), SLYNTVA**V**L (8V), SL**F**NT**I**ATL (3F6I), SL**F**NTVA**V**L (3F8V), SLYNT**I**A**V**L (6I8V) and SL**F**NT**I**A**V**L (3F6I8V). The high affinity 868 TCR (a11b6 TCR), or these mutated α and β chains in combination with a wild type chain were used in this study. Kinetic binding constants were calculated using BIAcore™ BIAevaluation software.

A

HLA variant	KD values			
	868 TCR Equilibrium/K_{on}/K_{off}	a11b6 TCR	a868b6 TCR	a11b868 TCR
6I	126 / 68nM	151 pM	2.3nM	3.6 nM
Index (SL9)	180 / 150nM	365 pM	4.9nM	8.7nM
6I8V	198 / 167nM	454 pM	6.3nM	7nM
8V	287 / 385nM	1.7 nM	19nM	21nM
3F6I	1.2 / 1.2μM	5.4 nM	69nM	81nM
3F6I8V	3.6 / 2.7μM	27 nM	239nM	346nM
3F	4.1 / 2.9 μM	21 nM	252nM	353nM
3F8V	10.5 / 2.7μM	69 nM	718nM	1.2μM

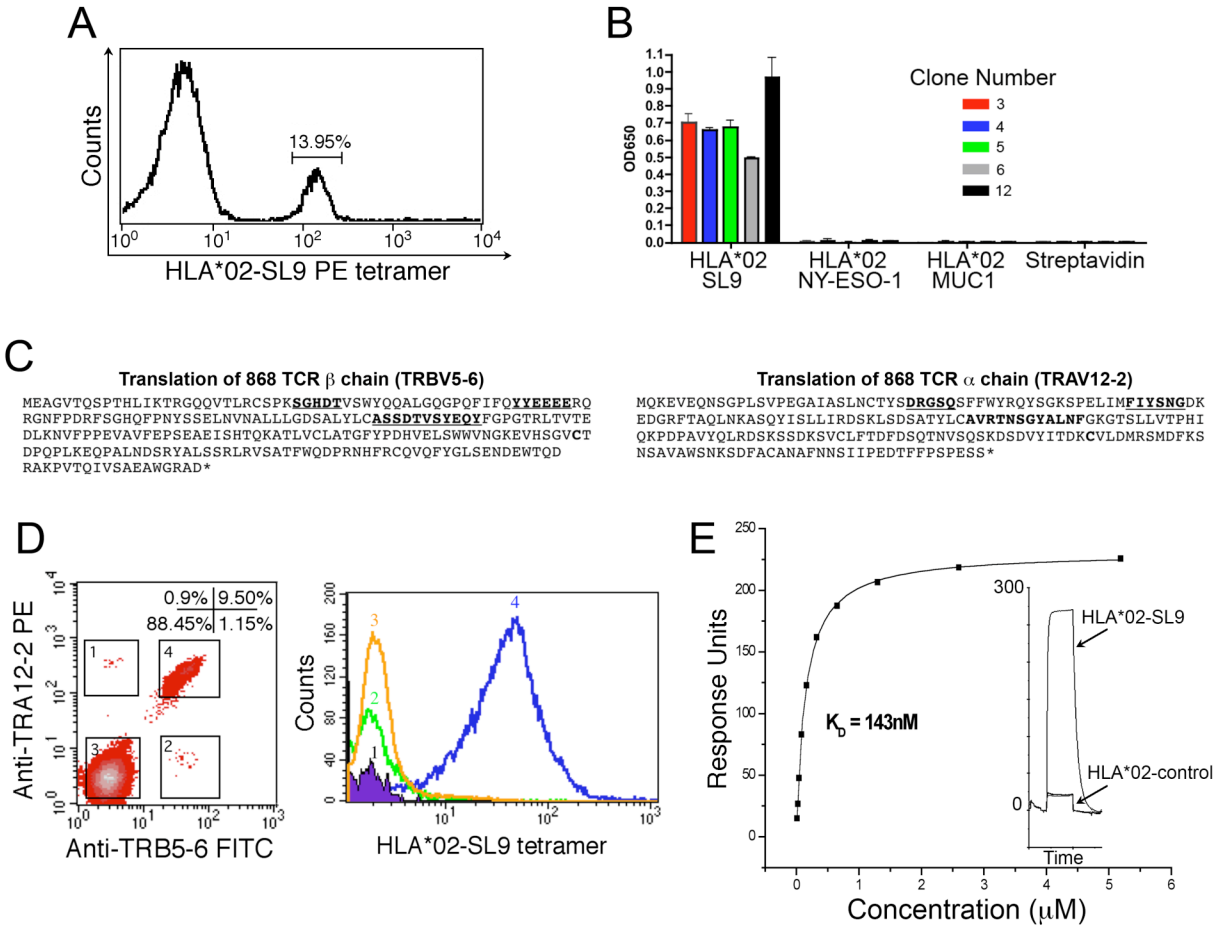
B

HLA variant	K_{on} values (1/Ms)			
	868 TCR	a11b6 TCR	a868b6 TCR	a11b868 TCR
6I	1.38×10^5	1.86×10^5	2.11×10^5	1.77×10^5
Index (SL9)	1.48×10^5	1.95×10^5	2.02×10^5	1.73×10^5
6I8V	1.38×10^5	2.95×10^5	2.77×10^5	2.18×10^5
8V	1.24×10^5	1.79×10^5	2.4×10^5	1.98×10^5
3F6I	1.19×10^5	1.84×10^5	1.83×10^5	1.89×10^5
3F6I8V	1.35×10^5	1.41×10^5	1.4×10^5	1.1×10^5
3F	1.48×10^5	1.53×10^5	1.62×10^5	1.43×10^5
3F8V	1.35×10^5	1.31×10^5	1.36×10^5	1.12×10^5

C

HLA variant	K_{off} values (1/s)			
	868 TCR	a11b6 TCR	a868b6 TCR	a11b868 TCR
6I	9.23×10^{-3}	2.81×10^{-5}	4.89×10^{-4}	6.45×10^{-4}
Index (SL9)	0.0222	7.12×10^{-5}	9.98×10^{-4}	1.5×10^{-3}
6I8V	0.0231	1.34×10^{-4}	1.75×10^{-3}	1.53×10^{-3}
8V	0.0534	2.97×10^{-4}	4.53×10^{-3}	4.06×10^{-3}
3F6I	0.148	9.83×10^{-4}	0.0127	0.0153
3F6I8V	0.36	3.78×10^{-3}	0.0335	0.0381
3F	0.428	3.18×10^{-3}	0.0408	0.0506
3F8V	1.16	0.0117	0.0976	0.13

(ii) Supplementary Figures



Supplementary Figure 1. Isolation and characterization of a HLA A2-SL9 specific TCR

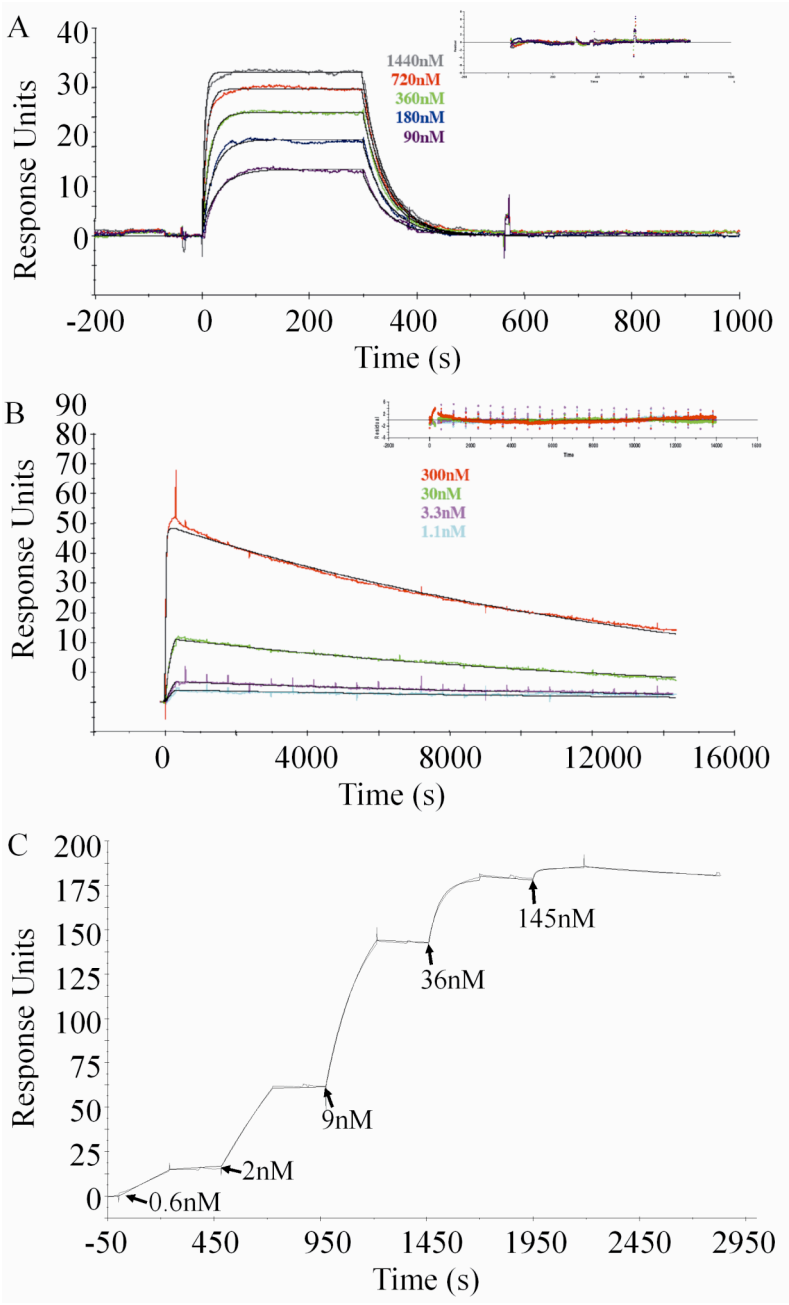
A) HLA A*02-SL9 tetramer staining of the of the 868 CTL. 868 CTL were stained in 20μl of PBS with 1 μg to HLA-A*02-restricted SL9 tetramers for 20 minutes at 37°C.

B) Several random clones from the Pan 3 HLA A*02-SL9 phage display selection were used to produce TCR bearing phage. These phage were screened for binding to HLA A*02-SL9 by using a phage ELISA. HLA A*02-NY-ESO, HLA-A*02-MUC1 and streptavidin were used as negative controls. A selection of the clones which gave a positive signal in the phage ELISA are shown in the histogram, none of the HLA A2-SL9 binding phage show cross-reactivity with the control HLAs or streptavidin.

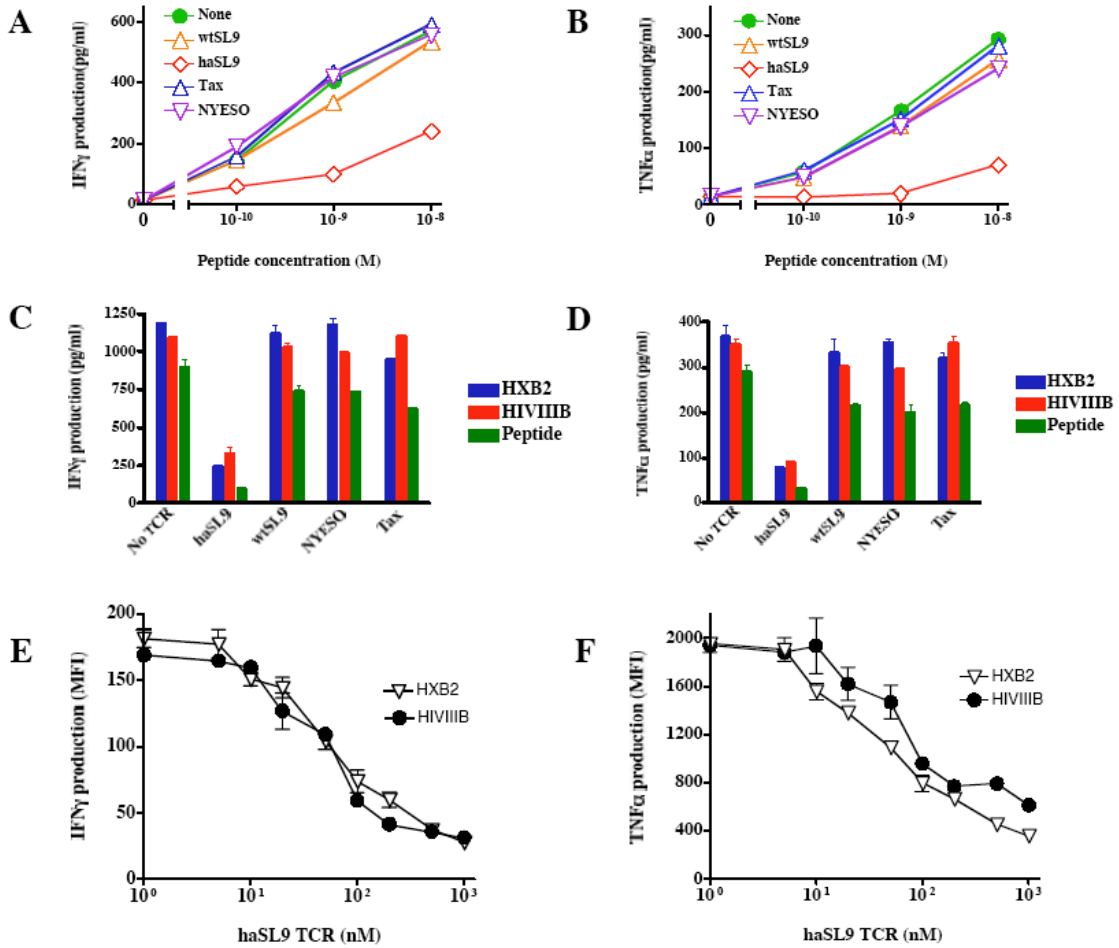
C) The translation of the TCR sequences derived from SL9-specific phage clone 4 (see B) is shown here. The beta chain is of the TRBV5-6 (IMGT nomenclature) family with a TRBJ2-7 J region. The alpha chain is of the TRAV12.2 family with TRAJ41 J region. These sequences are the exact sequences which were reconstituted into soluble TCR as described in Boulter, et al. 2003 *Protein Eng* **16**, 707-11. This method relies on the introduction of a non-native inter chain disulphide bond between the TCR alpha and beta chain constant regions, the introduced C residues are highlighted with in bold. The CDR regions of the TCR chains are underlined and highlighted in bold.

D) Expression of TRAV12.2 (Vα2) and TRBV5-6 (Vβ5) TCR chains by the 868 CTL line**.** The 868 line was stained simultaneously with 1 μg/ml of HLA-A*02-SL9 tetramer conjugated with APC, 5 μg/ml of anti-TCR Vα2 (clone F1) and Vβ5 (clone 1C1) antibodies (Endogen) for 20 min on ice. Cells were washed once in cold FACS buffer and 5 mg/ml of secondary antibodies were added. 7-Amino-actinomycin D staining was used to gate out dead cells. TRAV12.2 and TRBV5-6 chain expression pattern of the 868 line is shown (left panel D). Fraction of cells in each gate as a percentage of cells in all gates is shown inset. Only the cells expressing TRAV12.2 and TRBV5-6 (gate 4) stained with tetramers (right panel D).

E) BIAcore™ SPR analysis of the binding of purified SL9-specific TCR (clone 4) to HLA-A*02-SL9. Response values were calculated by subtracting the non-specific to HLA-A*02- ILAKFLHWL peptide from the specific response. Response readings were plotted against protein concentration calculated from absorbance at 280 nm using an extinction coefficient derived from the amino acid sequence. The K_D was calculated using a least-squares fit to the data assuming a 1:1 interaction. (Inset) A sample chromatogram derived from the BIAcore™ analysis of 868 TCR is shown. Binding of the TCR to the specific HLA-A*02-SL9 and non-specific HLA-A*02-ILAKFLHWL peptide as well as a blank flow cell are shown.

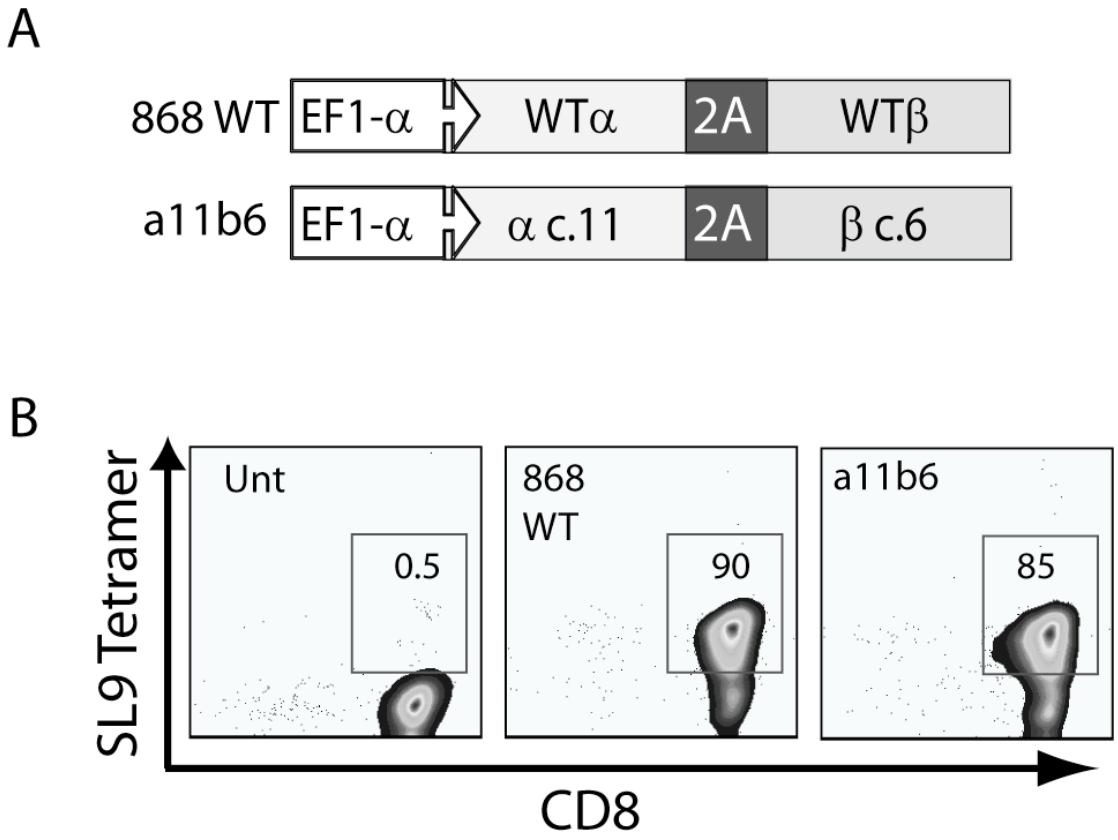


Supplementary Figure 2. Kinetic binding analysis of the 868 and a11b6 TCR interactions with HLA A2-SL9 – **(A)** Kinetic binding analysis of the 868 TCR to HLA A2-SL9. An equilibrium binding constant (K_D) of 150nM was calculated for the 868 TCR with an association rate (k_{on}) of 1.5 x 10^5 M^{-1} s^{-1} and a dissociation rate (k_{off}) of 2.2x10^{-2} s^{-1}. A range of concentrations were used for the analysis of the TCRs as indicated in the figure. The solid lines for each binding response were calculated assuming 1:1 Langmuir binding (($y = (AB = B*AB_{MAX}/(K_D + B))$ and the data were analyzed using global fitting. The residuals are shown in the top right hand corner panel of this experiment. **(B)** Kinetic binding analysis of the a11b6 TCR to HLA A2-SL9. A K_D of 365pM was calculated for the a11b6 TCR with a k_{on} of 1.95x10^5 M^{-1} s^{-1} and a k_{off} of 7.1x10^{-5} s^{-1}. A range of concentrations were used for the analysis of the TCR as indicated in the figure. The solid lines for each binding response were calculated assuming 1:1 Langmuir binding (($y = (AB = B*AB_{MAX}/(K_D + B))$ and the data were analyzed using the global fits generated. The residuals are shown top right hand corner panel of this experiment. **(C)** Kinetic titration analysis for the a11b6 TCR. This analysis was carried out using state-of-the-art BIAcore T100™ technology in order to confirm the accuracy of the data from the analysis in **(B)**. The concentration at each injection point is shown in the figure. A K_D of 245pM was calculated for the a11b6 TCR with a k_{on} of 2.2x10^5 M^{-1} s^{-1} and a k_{off} of 5.4x10^{-5} s^{-1} The solid line for the binding response was calculated assuming 1:1 Langmuir binding (($y = (AB = B*AB_{MAX}/(K_D + B))$ and the data were analyzed using a kinetic titration analysis algorithm (BIAevaluation™ 3.1) to calculate K_{on}, K_{off} and K_D values.



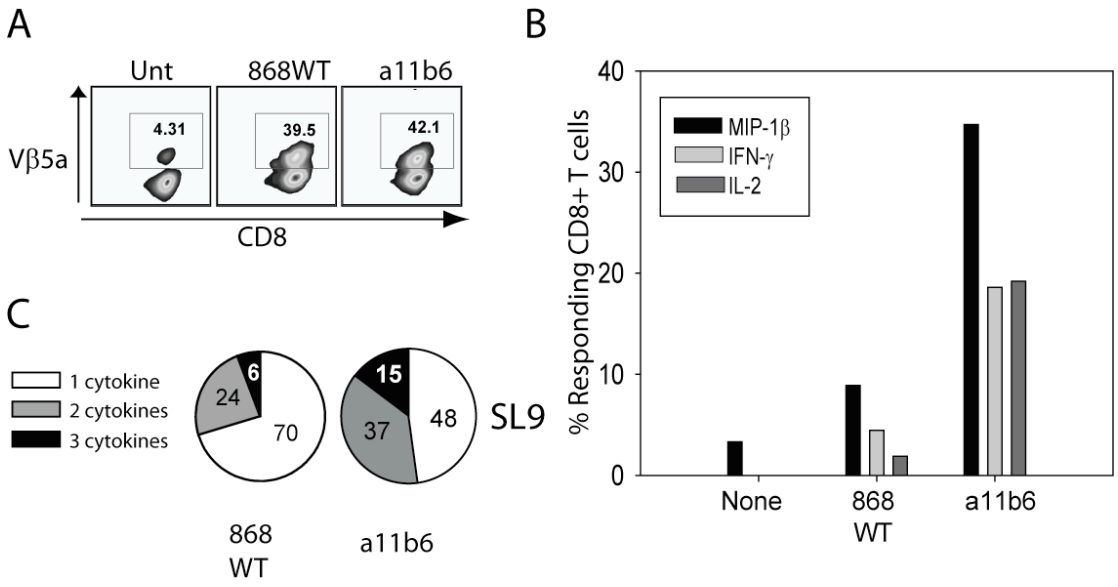
Supplementary Figure 3. High affinity Gag TCR inhibits SL9-specific cytotoxic T cells.
T_0 cells were either chronically infected with HIV_{IIIB} or HXB2 HIV strains or pre-pulsed with SL9 synthetic peptide at concentrations indicated in figure legends. The pre-pulsing was done by incubating the cells in R10 medium (RPMI 1640 containing 10% foetal calf serum, 100 U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine) containing peptide for 1h at room temperature and then washing once in R10. The pre-pulsed or HIV-infected T_0 cells were then used as targets for a SL9-specific CTL clone in an ELISA assay. Prior to the addition of the CTL, the TCR was added to the targets at a final concentration of 200nM (or as indicated in titration experiments) and incubated at 37ºC for 30 minutes. Targets and CTL were used at 1:1 ratio (25,000 of each) and assays were carried out in a 96-well U-bottom plate in 100ml total volume. Following a 4-hour incubation at 37ºC, the supernatant was harvested and assayed for cytokine production using the CBA cytokine kit (BD Biosciences), according to the manufacturer's instructions.

A&**B**: a11b6 TCR-blocking of IFNγ (**A**) and TNFα (**B**) production by an SL9-specific CTL clone in response to HLA $A2^+$ targets pulsed with different concentrations of SL9 peptide. Wild-type SL9 specific TCR (wtSL9) and two irrelevant enhanced affinity TCRs (HLA-A*02 Tax-specific A6c134 and HLA-A*02 NY-ESO-1-specific 1G4c113) were used as controls. (a11b6 TCR did not inhibit the activity of CTL specific for two other HIV epitopes: HLA-A*02 -restricted HIV reverse transcriptase epitope ILKEPVHGV and HLA-B*08-restricted HIV Nef epitope FLKEKGGL; data not shown). **C**& **D**: a11b6 TCR blocking of IFNγ (**C**) and (**D**) TNFα production by a SL9-specific CTL clone in response to T_0 targets infected with two HIV strains (HIV_{IIIB} and HXB2) or pulsed with the SL9 peptide at 10^{-9}M. **E**&**F**: titrating the inhibitory effect of the a11b6 TCR on IFNγ (**E**) and (**F**) TNFα production by SL9-specific CTL using T_0 targets infected with HXB2 and HIV_{IIIB}.



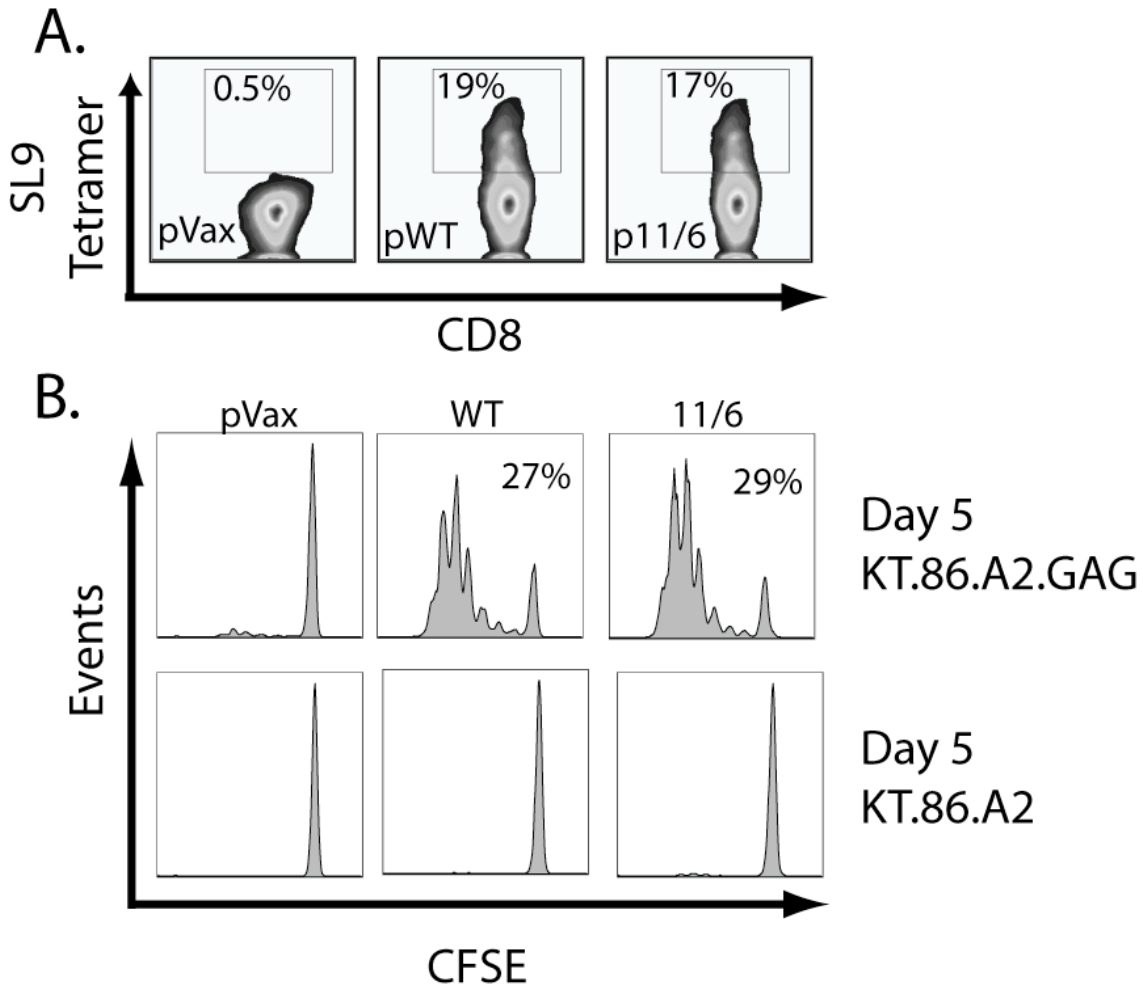
Sup Figure 4

Supplementary Figure 4. SL9-transduced CD8 T cells recognize SL9 tetramer. Primary human CD8 T cells were transduced with the indicated SL9 specific TCRs and stained with PE labeled SL9 tetramer 8 days after transduction.



Sup Figure 5

Supplementary Figure 5. CD8 T cells expressing supraphysiologic SL9-specific TCRs show an increased frequency of polyfunctional cells. A. CD8 T cells were transduced with the SL9 TCR constructs and stained with Vβ5α Ab which recognizes the introduced SL9-specific TCRs. **B.** Cells described in **A.** were stimulated by K562 cells expressing HLA-A*0201 (KT.A2) loaded with 50 pg/ml SLYNTVATL (SL9) peptide expression of IFN-γ, IL-2 and MIP-1β was measured by intracellular cytokine staining (Data shown in Fig. 2). The percentage of CD8 T cells producing MIP-1β (black), IFN-γ (white) and IL-2 (gray) after SL9 KT.A2 stimulation was measured is displayed **C.** The number of triple-function responses (expressing MIP-1α, IFN-γ and IL-2) are coded as black, dual-function cells are coded as grey, and single-function responses are coded as white. Results are representative of three independent experiments.



Supplemental Figure 6. CD8 T cells transduced with 868 and a11b6 TCRs exhibit similar levels of proliferation when exposed to HLA A*02+ targets expressing the HIV Gag gene. **A**. CD8 T cells were transfected with an empty pVAX vector or pVAX vectors expressing SL9 specific TCR constructs. Staining with SL9 tetramer 18 hours after transfection showed similar levels of transfection. **B**. SL9-specific TCR transfected CD8 T cells were labeled with CFSE prior to stimulation with KT.64.86.A2. or KT.64.86.A2.GAG aAPCs. CFSE dilution was measured after 5 days of culture. The frequency of responding cells for each histogram is shown. Data is representative of four independent experiments.

(iii) Supplementary Methods

Phage Libraries and Selections

RNA was prepared from the 868 line using the Amersham Quick Prep Micro mRNA kit and used to generate cDNA. Comprehensive TCR primer sets were designed with reference to the known human TCR sequences families such that the Vα and Vβ chains present in the 868 line cDNA could be amplified by PCR. Pooled Vα and pooled Vβ amplification products were subjected to a second round of PCR using common flanking primers in order to introduce sufficiently long constant domain tags. These pools of Vα/Vβ chains were randomly assembled into pairs using a splicing reaction employing common intervening fragments derived from the destination phagemid vector (the vector has previously been described in Li et al. *Nat Biotechnol* **23**, 349-354). The spliced products were cloned into the destination phagemid vector and electroporated into *E. coli* TG1 to generate a library of 10^8 clones. Phage bearing TCR were generated by standard protocols and subjected to three rounds of panning against biotinylated HLA A2-SL9. Third round clones were screened by phage ELISA to determine binding to HLA A2-SL9. HLA A2-NY-ESO and HLA A2-MUC1 were used as negative controls in the phage ELISA.

The parental phagemid (clone 4) containing the native α and β chain was used as a starting point for the construction of CDR2 (Dunn, S.M. et al. 2006 *Protein Sci* **15**, 710-21) and CDR3 mutated phage display libraries. Briefly, CDR2α mutations were introduced into the α chain by a 2-fragment PCR splicing technique with 4 x NNK codons substituting for the parental germline amino acid sequence IYSN. Similarly, a CDR2β library was constructed by randomization of the parental sequence YEEE with 4 x NNK codons. For the CDR3 libraries, mutations were introduced across the 7aa core loop regions (RTNSGYA for CDR3α; SDTVSYE for CDR3β) using a pooled panel of 7 mutagenic oligo's designed to bias the mutation space towards conservative substitution of the parent codons (according to the neutral and positive scoring substituents of the Blosum-62 matrix), whilst allowing adequate wildcard/random diversity. Such a strategy allows for greater reach across the loop than could be practically achieved with NNK-based randomization due to library-size limitations. Following electroporation of the mutated phagemid DNA into *E.coli* strain TG1, CDR2 libraries of 10^7 and CDR3 libraries of $1.5x10^8$ were achieved. TCR-displaying phage particles were subsequently rescued from the libraries using M13KO7 helper phage according to standard protocols. Phage isolated from the CDR2 libraries was pooled prior to selection as was CDR3 phage. A streptavidin magnetic bead-based selection procedure, similar to that employed for the initial isolation of the clone 4 parent, was performed and output clones screened for binding using a standard microwell format alkaline-phosphatase ELISA.

Soluble protein production and affinity measurement

Soluble high affinity TCRs were produced as disulfide-linked heterodimeric TCRs, as described in Boulter, et al. 2003 *Protein Eng* **16**, 707-11. In all cases, the soluble proteins were gel filtered into HBS buffer on the day of analysis in order to minimize potential protein aggregation. Biotinylated and non-biotinylated peptide-HLA complexes were prepared as described in O'Callaghan C, A. et al. 1999 *Anal Biochem* **266**, 9-15. We conducted surface plasmon resonance (SPR) analysis of variant TCRs binding to biotin-tagged HLA A2-SL9, immobilized to a streptavidin-coated flow cell using a BIAcore 3000™, or a BIAcore T100™. An identical amount of HLA A2-NY-ESO-1 was immobilized onto one flow cell as a negative control. A relatively low amount of ligand (~100 RUs) was coupled to each flow cell in order to minimize the effects of mass transport during the dissociation period, as advised by BIAcore™. All experiments were carried out at a flow rate of 50 μl/min in order to minimize mass transport effects during the association phase. For the 868 TCR, an association period of 300 secs and a dissociation period of 700 secs were used for the analysis. Kinetic binding constants were calculated assuming 1:1 Langmuir binding (($y = (AB = B*AB_{MAX}/(K_D + B)$) by using a global fitting algorithm (BIAevaluation™ 3.1). For the a11b6 TCR, an association period of 300 secs was used to analyze the k_{on}. Dissociation phase data was collected for at least 5 hours to allow accurate determination of the extremely slow k_{off} values. Equilibrium binding constants for TCRs exhibiting slow off-rates were calculated from kinetic data ($K_D = k_{off}/k_{on}$). Between each injection of the a11b6 TCR at each concentration, the chip surface was completely stripped of streptavidin using BIAcore desorb solution 1™ (0.5% SDS) and BIAcore desorb solution 2™ (50 mM glycine, pH 9.5). This chip surface was then re-activated, re-coated with streptavidin and fresh biotin-tagged HLA A2-SL9 was immobilized to the streptavidin-coated flow cell for subsequent a11b6 TCR injections. Kinetic binding constants were calculated using BIAevaluation™ software, by separately analyzing each injection of TCR, assuming 1:1 Langmuir binding (($y = (AB = B*AB_{MAX}/(K_D + B)$) by using a global fitting algorithm (BIAevaluation™ 3.1).

SPR Kinetic Titration Analysis

In order to more stringently examine the binding of the a11b6 TCR at a greater range of concentrations, we used BIAcore T100™ technology. In all cases, the soluble proteins were gel filtered into HBS buffer on the day of analysis. Due to the extremely low concentration of the injections used during the analysis, the a11b6 TCR did not require pre-concentration and the absorbance 280nm was measured in triplicate using neat sample. These steps insured accurate

measurements of the protein concentration of the samples and minimized the potential for protein aggregation to occur. The a11b6 TCR was analyzed at 5 concentrations ranging from 145nM to 0.6nM. These concentrations represent the greatest range we could accurately achieve near the K_D for this interaction. During the analysis, ~200 RUs of pMHC were immobilized onto the CM5 sensor chip surface. Each concentration of TCR was injected at a high flow rate of 45µl/min, and a data collection rate of 10 Hz, for a 240 sec association period and a 120 sec dissociation period. The final and highest concentration had a longer dissociation period of 600 sec. A fast flow rate and a low amount of immobilized pMHC were used in order to limit association and dissociation mass transfer limitations. The K_{on} and K_{off} values were calculated assuming 1:1 Langmuir binding ((y = (AB = B*AB_{MAX}/(K_D + B)) and the data were analyzed using the kinetic titration analysis algorithm (BIAevaluation™ 3.1) (Karlsson, et al. 2006 *Analytical biochemistry* **349**, 136-147).

TCR Constructs

Wildtype TCR α and β genes were cloned from a SLYNTVATL specific T cell line grown from an elite non-progressor, patient 9300868 (Sewell et al. 1997 *Eur. J. Immunol.* **27**, 2323-2329). High affinity mutants were screened by phage display and isolated as previously described Dunn *et al.* 2006 *Protein Sci.* **15**, 710-721. 868a, 868b, clone a11 and clone b6 genes were codon-optimized (Geneart, Regensburg, Germany) and subcloned into four different pairs of alpha and beta genes in pVax (Invitrogen) via a T2A sequence containing a GSG linker (Szymczak et al. *2004 Nat. Biotechnol.* **22**, 589-594). The fragments encoding the alpha-2A-beta pairs were subcloned into the pELNS lentiviral transfer plasmid for lentiviral vector production. All constructs were confirmed by DNA sequencing.

TCR lentiviral vector production, CD8 T cell and SupT1 transductions

High titer lentiviral vectors were produced as described previously (Parry el al. 2003 *J. Immunol.* **171**, 166-174). CD8 T-cells were stimulated for 24 hrs with anti-CD3/anti-CD28-coated Dynal beads (Invitrogen) before transduction. Five hundred µl of concentrated lentiviral vector was incubated with 0.5-1 x 10^6 activated T-cells on 24-well plates at 37°C. The medium was changed the following day and the cells expanded until they returned to near resting volume (between 8-12 days), as measured in a Multisizer-3 (Beckman-Coulter, Miami, FL). ~~The T-cell line SupT1 was transduced by incubating 5 X 10^5 cells with 500 µL of concentrated lentiviral vector. After an overnight incubation, the cells were washed once with fresh medium and resuspended in 1 mL complete RPMI 1640.~~ Transduction efficiencies were assessed by flow cytometric analysis of TRBV5-6 (Vb5a) staining (Pierce Biotechnology, Rockford, IL) or HLA A2-SL9 tetramer stain (Beckman Coulter Immunomics, San Diego, CA).