

Targeting peptide antigens using a multiallelic MHC I-binding system

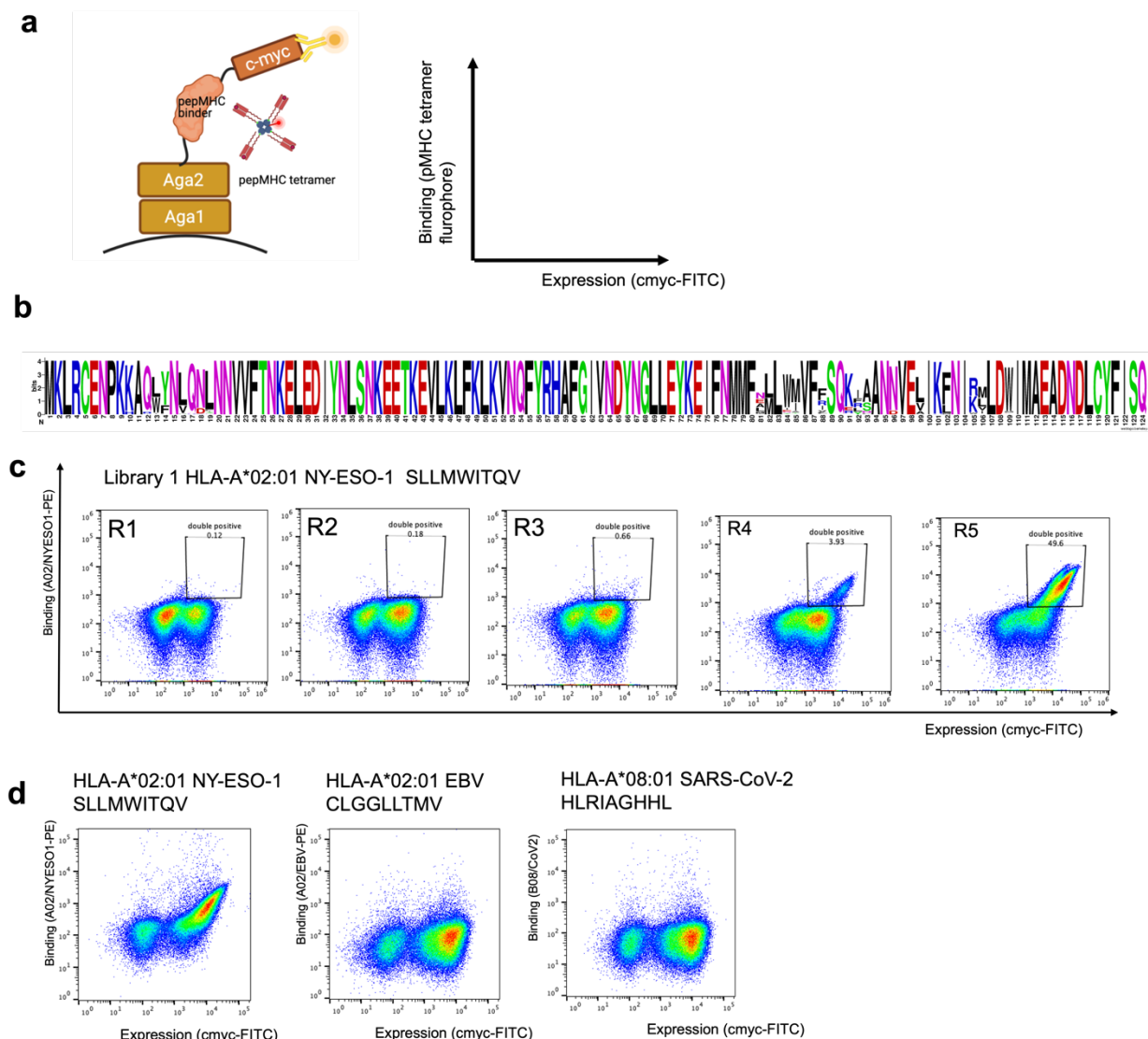
In the format provided by the
authors and unedited

Table of Contents

<i>Figures:</i>	3
Supplementary Figure S1. Library 1 design and selection for MHC-I <i>TRACeR</i>	3
Supplementary Figure S2. <i>TRACeR</i> binding profile with ARE depletion or disulfide bond depletion	4
Supplementary Figure S3. Analysis of polymorphic MHC-I residues relative to <i>TRACeR</i> contact positions.....	5
Supplementary Figure S4. Library 2 (ARE library) selection for multiple MHC-I targets	6
Supplementary Figure S5. Circular permutation scheme for redesigning <i>TRACeR</i> scaffold	7
Supplementary Figure S6. Specificity characterization for <i>TRACeRMHC – I, A02NY – ESO – 1</i> and <i>TRACeRMHC – I, A02EBV</i> on BLI	8
Supplementary Figure S7. Protein purification for <i>TRACeR</i> constructs	9
Supplementary Figure S8. Initial assessment of <i>TRACeR</i> specificity is performed by binding test with a random peptide library consisted of 50 peptides from TCGA library	10
Supplementary Figure S9. Staining and selection process on the triplet scanning library	11
Supplementary Figure S10. SSM analysis on the triplet scanning library	12
Supplementary Figure S11. Peptide selection of <i>TRACeRMHC – I, A02NY – ESO – 1</i>	13
Supplementary Figure S12. <i>In vivo</i> and <i>ex vivo</i> evaluation of immunogenicity of <i>TRACeRMHC – I, A02NY – ESO – 1</i> (n=8 mice/group).....	15
Supplementary Figure S13. <i>TRACeR</i> do not produce significant long-term T cell responses and do not retain mitogenic activity.	16
Supplementary Figure S14. Immune cell activation profile under <i>TRACeR</i> treatment	18
Supplementary Figure S15. <i>In vitro</i> tumor cell killing assays with control BiTEs.....	19
Supplementary Figure S16. SEC trace of the co-purification of the <i>TRACeRMHC – I A02NY – ESO – 1</i> /HLA-A*02:01/NY-ESO-1 complex.	20
Supplementary Figure S17. Structure analysis of <i>TRACeRMHC – I, A02NY – ESO – 1</i>	20
Supplementary Figure S18. Electron density omit maps shows interface of <i>TRACeR</i> -HLA-A*02:01/β2m/NY-ESO-1 complex.....	21
Supplementary Figure S19. Comparison of binding modes between TCR, Fab and <i>TRACeR</i> interacting with the same NY-ESO-1 peptide	22
Supplementary Figure S20. Purification and affinity measurement of <i>TRACeRMHC – I, A02NY – ESO – 1</i> with improved specificity	23
Supplementary Figure S21. Purification and binding specificity of rewired monomeric <i>TRACeR</i>	24
Supplementary Figure S22 Specificity of the rewired monomeric <i>TRACeR</i> determined by the yeast displayed triplet scanning library	26
Supplementary Figure S23 Receptor design, expression and tetramer binding of engineered T cell receptors	27

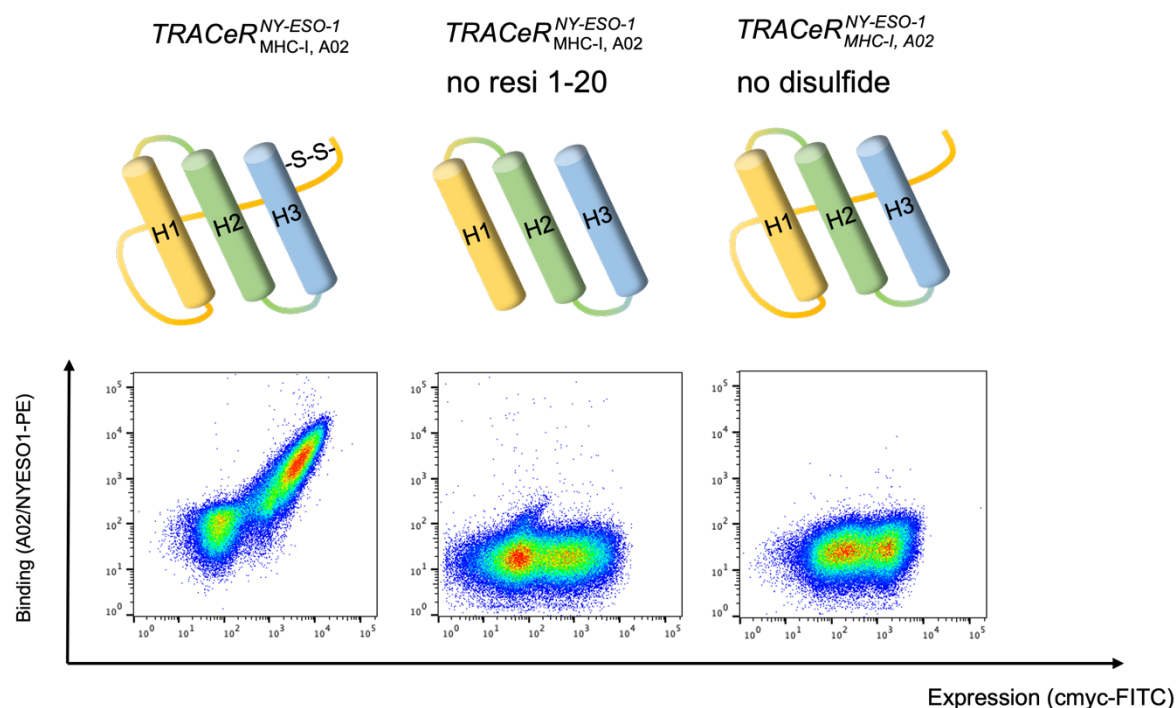
Supplementary Figure S24. In vitro tumor cell killing assays with TRACeRMHC – I, A02NY – ESO – 1 -CAR T cells in comparison with a CAR implemented with a TCR (1G4) as the binding domain.	28
Supplementary Figure S25. Enrichment process for all 6 targets from the master library	32
Supplementary Figure S26. Protein purification and characterization for <i>TRACeRs</i> selected from the monomeric master library	33
Supplementary Figure S27. <i>TRACeRs</i> selected from the master library can detect single amino acid difference on the peptide	34
Supplementary Figure S28. SSM analysis of TRACeRMHC – I, A11KRAS_G12V with tetramer staining	35
Supplementary Figure S29. Comparison of sequence identities among all common alleles and alleles that are proven to be compatible with <i>TRACeR</i>	36
Tables	37
Supplementary Table 1. Summary of relevant amino acid sequences	37
Supplementary Table 2. Binding kinetics summary of dimeric <i>TRACeRs</i>	39
Supplementary Table 3. Predicted binding and thermal stabilities for exchanged HLA-A*02:01 bound to individual TCGA and NY-ESO-1 position 1, 4, 5, 7, and 8 full-scanning epitopes	40
Supplementary Table 4. Crystallography Data Collection and Refinement Statistics	45
Supplementary Table 5. Binding kinetics summary of monomeric <i>TRACeRs</i>	46
Supplementary Table 6. Sequence and convergence pattern for each target in the master library	46
Appendix	48
Supplementary Appendix 1. PDB ID info for Fig. 1A.....	48
Supplementary Appendix 2. PatchDock seeded RifDock flags setup for global docking step.....	48
Supplementary Appendix 4. RosettaScripts for running interactive docking and interface design.....	51
Supplementary Appendix 5. Script to filter Rosetta outputs on evaluation metrics and sort outputs based on binding energy	55
Supplementary Appendix 6. Peptide loading was confirmed for individual pHLA-A*02:01 tetramer..	56
References	58

Figures:



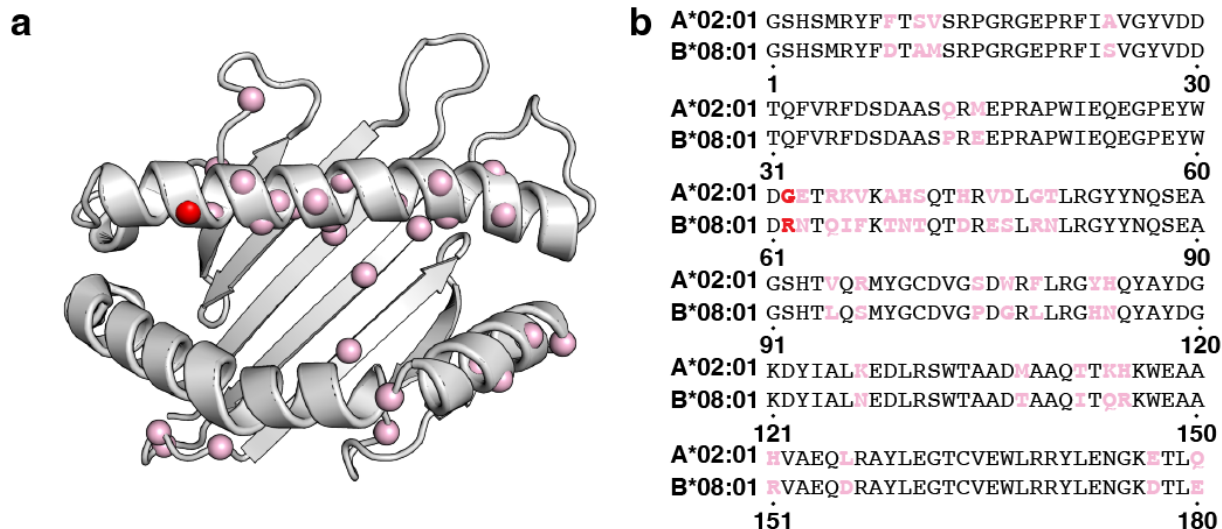
Supplementary Figure S1. Library 1 design and selection for MHC-I *TRACeR*

a. Schematic of the yeast surface display experiment. **b.** Library sequence design based on Rosetta sequence design and Swiftlib codon coverage. **c.** Screening the first combinatorial library constructed based on the design outputs. After 5 rounds of screening, a single clone of HLA-A*02:01/NY-ESO-1 binder was enriched from the pool. Staining concentration: Round 1-3: 250 nM MHC tetramer (1 μ M pMHC monomer + avidity effect), Round 4-5 100 nM MHC tetramer (400 nM pMHC monomer + avidity effect). **d.** The first enriched clone can differentiate pMHC targets, suggesting a binding mode that directly interact with the presented peptide.



Supplementary Figure S2. *TRACeR* binding profile with ARE depletion or disulfide bond depletion

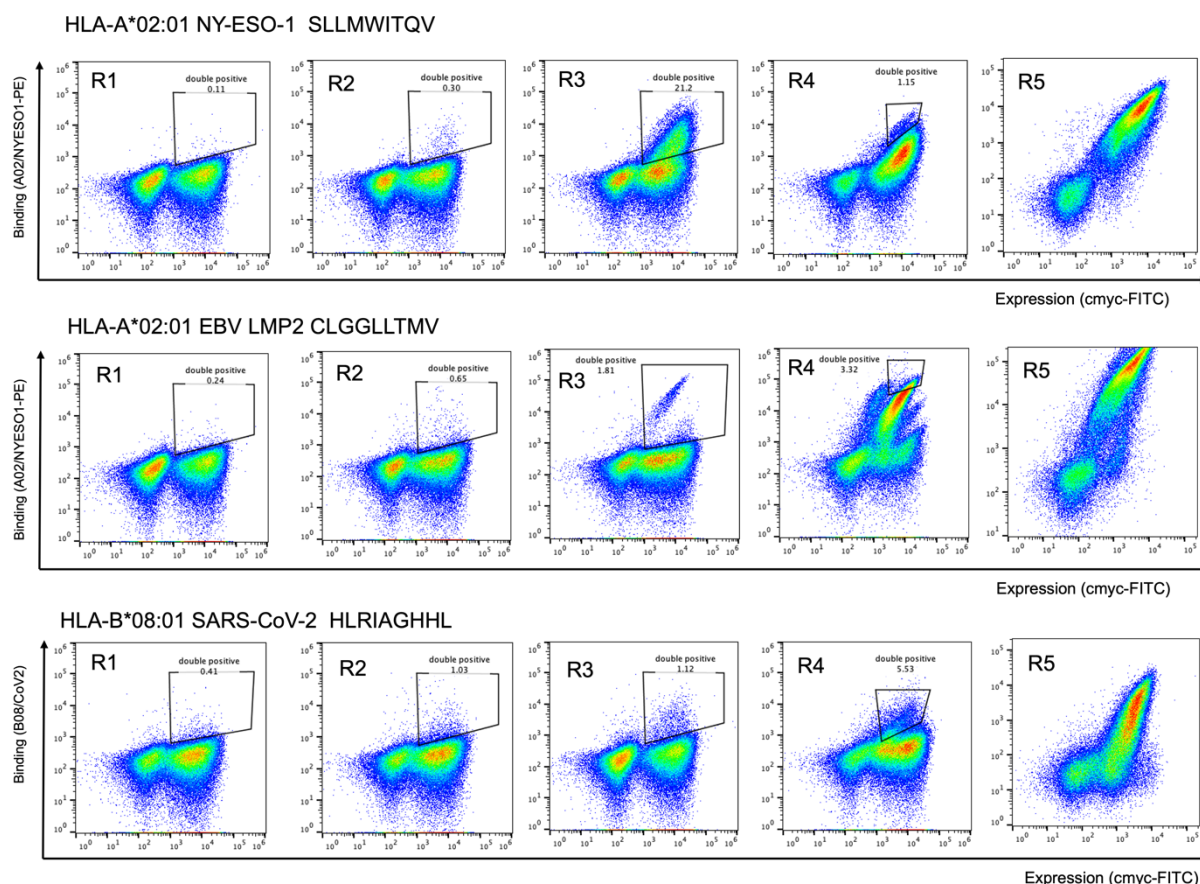
To support the structural hypothesis that the ARE region is essential for pMHC target binding and support the library 2 construction rationality. The *TRACeR* binding is profiled either with the ARE region depleted or with removal of the disulfide that is critical for ARE placement. The depleted binding signal further proved that the existence and proper placement of ARE is essential for *TRACeR*-pMHC binding. (Staining concentration: 50 nM pMHC tetramer)



Supplementary Figure S3. Analysis of polymorphic MHC-I residues relative to TRACeR contact positions

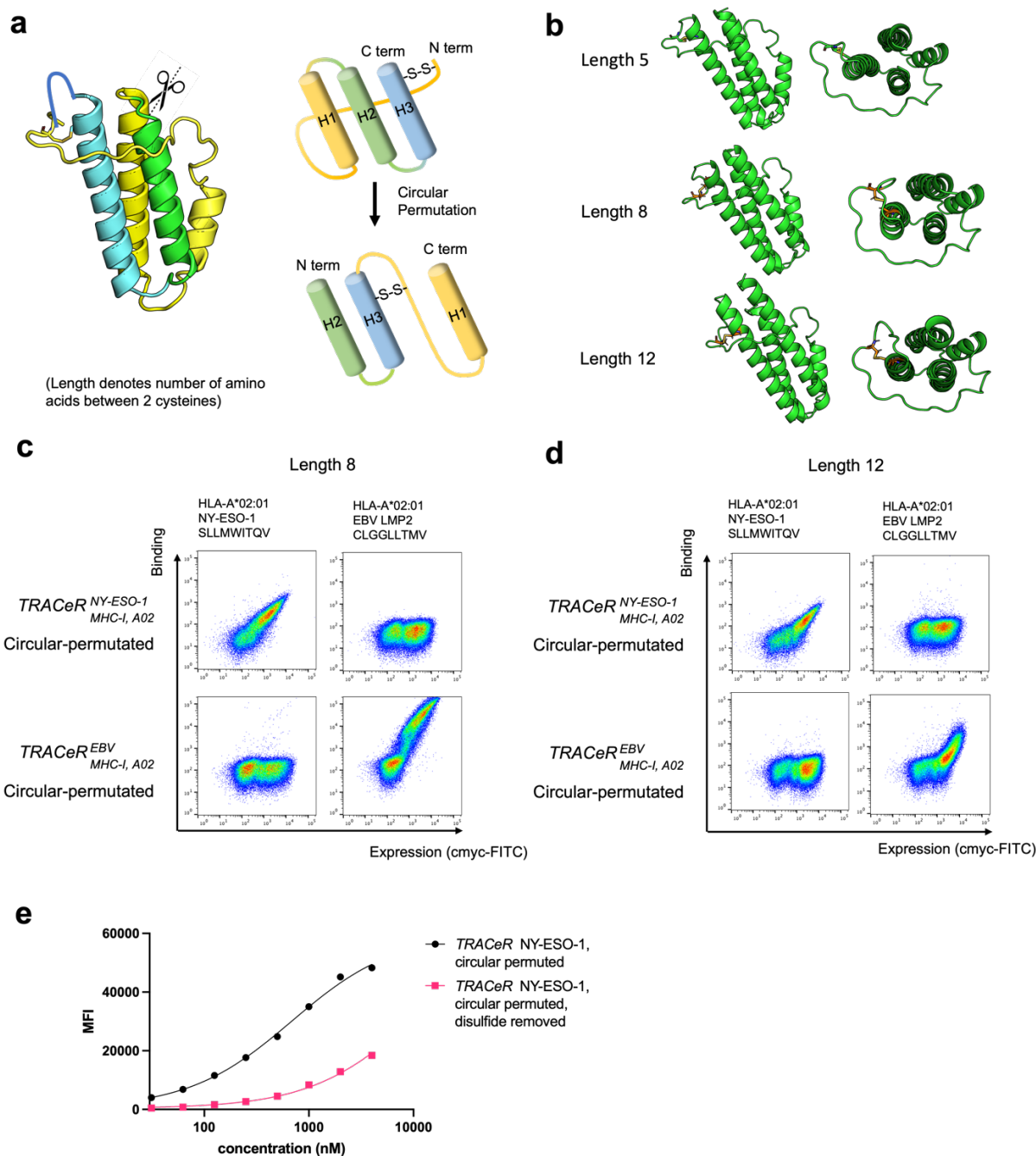
a. Polymorphic residues (pink spheres) between HLA-B*08:01 and HLA-A*02:01. The red sphere represents a single polymorphic residue at position 62 that forms part of the *TRACeR* interface in our A02/*TRACeR* complex structure. **b.** Sequence alignment of alpha1/alpha2 MHC-I domains between HLA-A*02:01 and HLA-B*08:01. All polymorphic positions are shown in pink, while *TRACeR* contact residues are shown in red.

Library 2



Supplementary Figure S4. Library 2 (ARE library) selection for multiple MHC-I targets

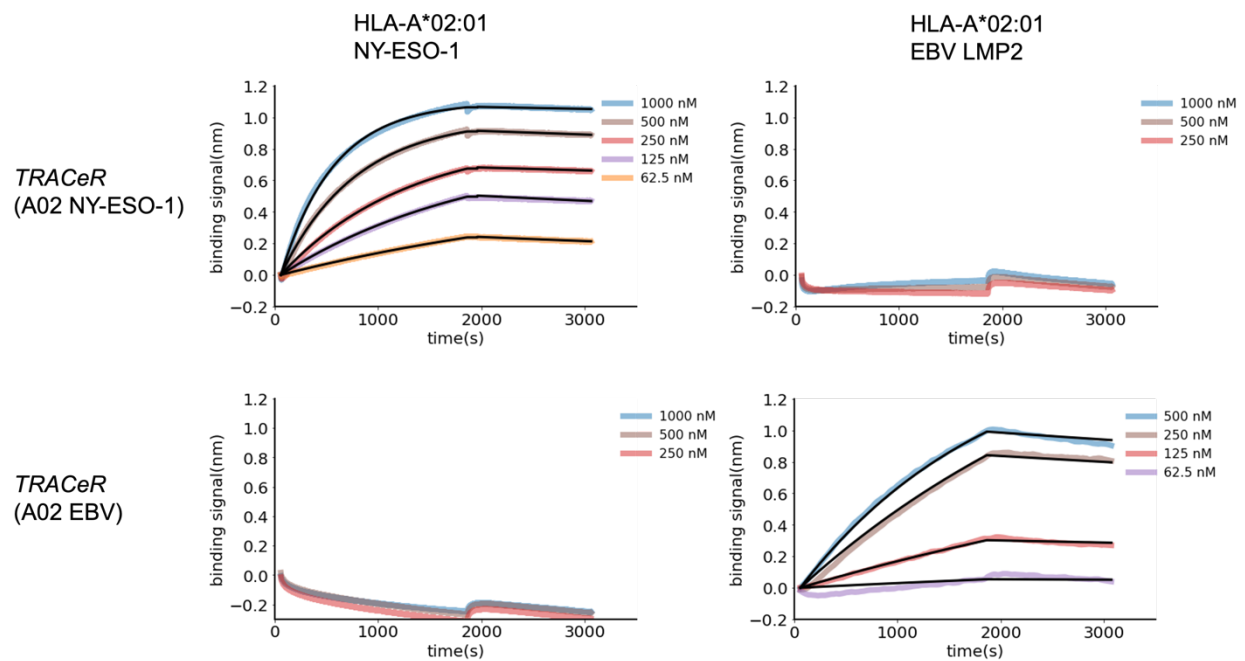
Library screening for different targets from the second library that mutates the ARE region on the first binding clone. Staining concentration: Round 1-3: 50 nM MHC tetramer (200 nM pMHC monomer + avidity effect), Round 4-5 25 nM MHC tetramer (100 nM pMHC monomer + avidity effect). For the library 2, *TRACeR* for NY-ESO-11₁₅₇₋₁₆₅ (SLLMWITQV) with higher binding signal under the same staining concentration was identified, showing that mutating the ARE region helped with the affinity maturation of the original *TRACeR*. The ARE library also expanded *TRACeR* binding to another two MHC-I targets.



Supplementary Figure S5. Circular permutation scheme for redesigning *TRACeR* scaffold

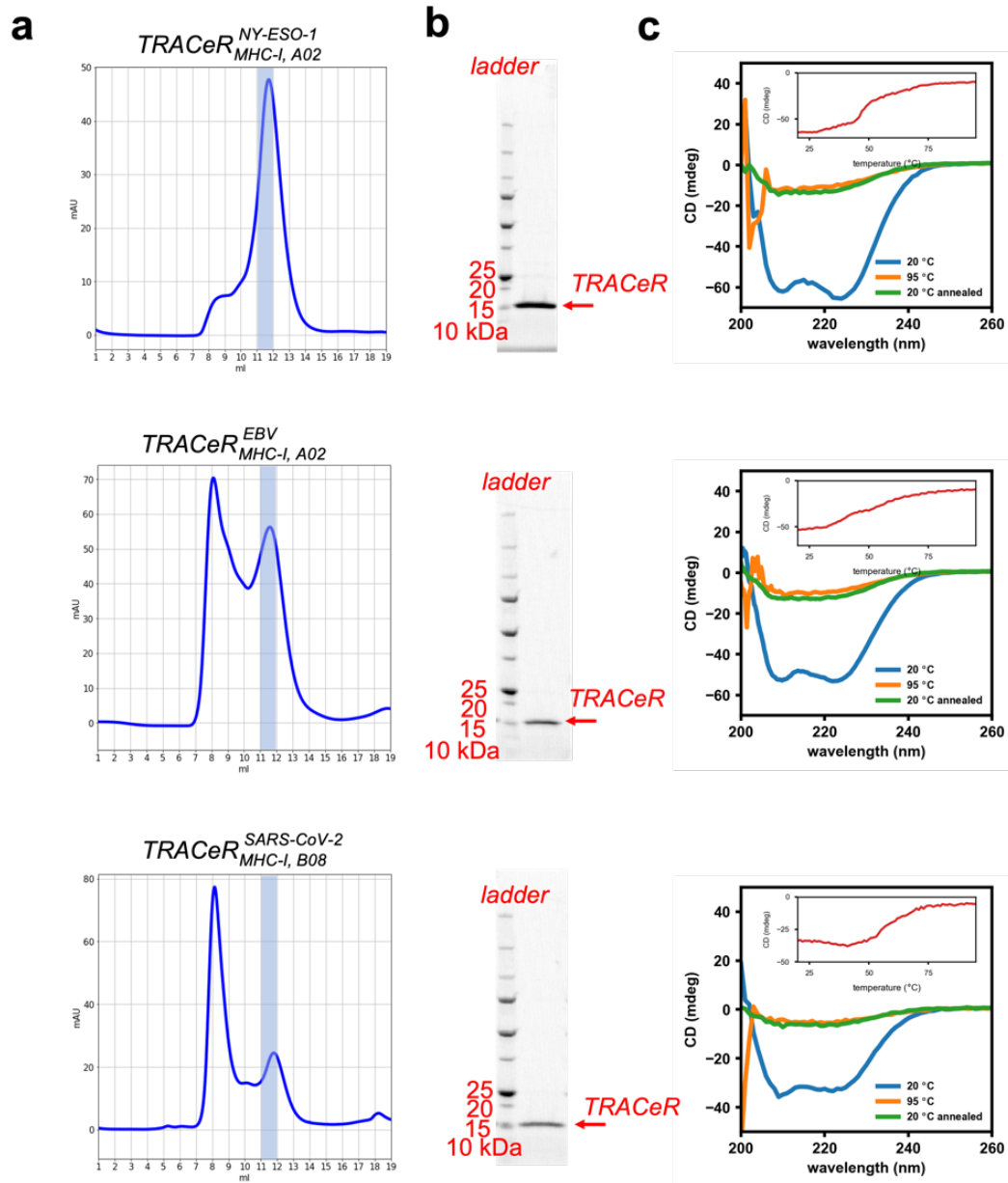
a. A circular permutation scheme is introduced to redesign the *TRACeR* scaffold to improve the stability and reduce the disulfide cross-link associated with the initial end-to-tail disulfide construct. The helices were reordered, and the original N and C terminus were connected by a redesigned loop. New N and C terminus is shown as “cut site”. **b.** Computational models for different loop lengths. Models are built with RosettaRemodel. Length 5, 8, 12 (defined by number of amino acids between the two disulfide cysteines) can result in proper loop closure. **c.** The length

8 circular permuted scaffolds retain the specificity pattern of $TRACeR_{MHC-I,A02}^{NY-ESO-1}$ and $TRACeR_{MHC-I,A02}^{EBV}$. **d.** The length 12 circular permuted scaffolds retain the specificity pattern of $TRACeR_{MHC-I,A02}^{NY-ESO-1}$ and $TRACeR_{MHC-I,A02}^{EBV}$. Both length 8 and length 12 scaffolds maintain the antigen specificity. Length 8 has higher binding signals for both $TRACeRs$, therefore was incorporated into the final $TRACeR$ design for affinity consideration. **e.** Titration of the circular permuted $TRACeR_{MHC-I,A02}^{NY-ESO-1}$ and with and without disulfide removal through Cysteine to Serine mutation. $TRACeR$ is displayed on yeast surface and titrated with A02/NY-ESO-1 pMHC monomer.



Supplementary Figure S6. Specificity characterization for $TRACeR_{MHC-I,A02}^{NY-ESO-1}$ and $TRACeR_{MHC-I,A02}^{EBV}$ on BLI

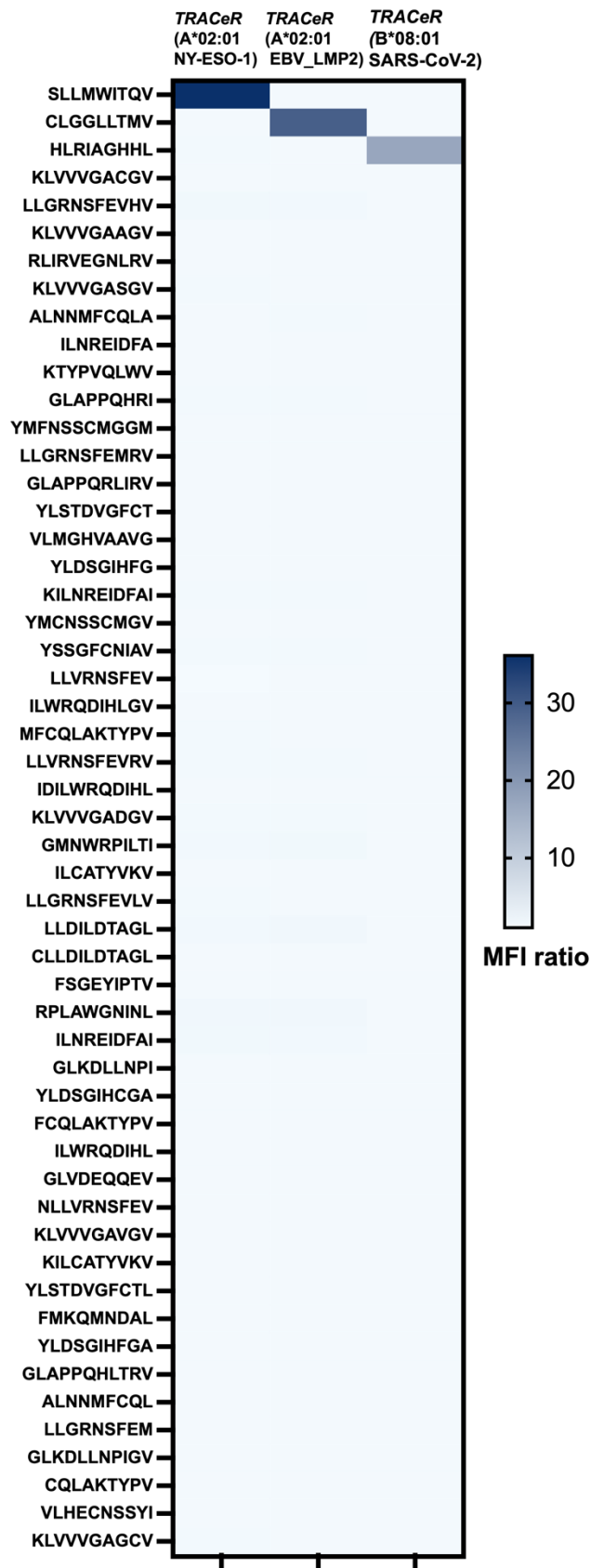
BLI measurement of $TRACeRs$ binding with on- and off-targets.



Supplementary Figure S7. Protein purification for *TRACeR* constructs

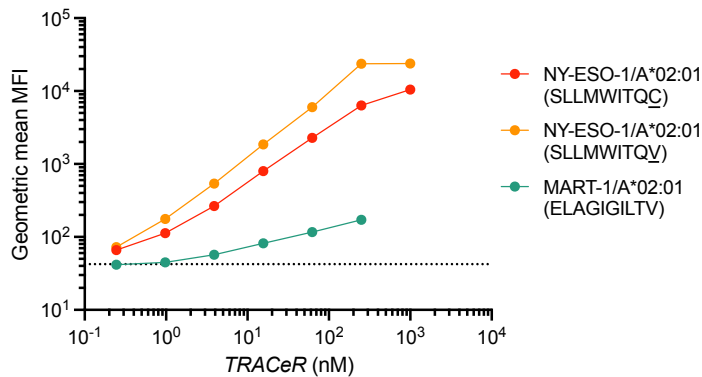
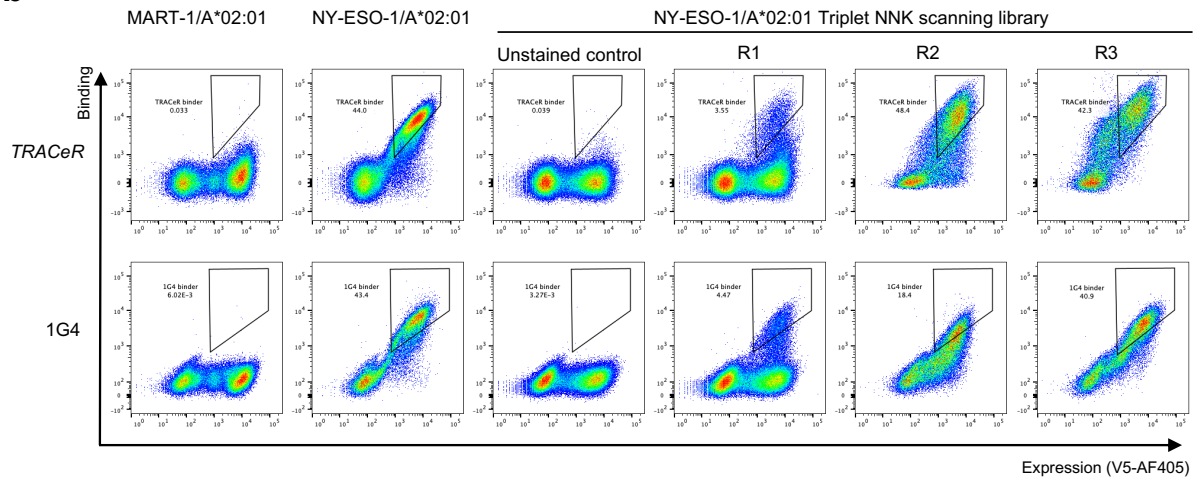
a. SEC plot of *TRACeR* constructs purification after purifying with Nickel column. Collected elution peak for downstream characterization and affinity determination is colored in shadow. **b.** The SDS-PAGE gel for SEC purified *TRACeR*. Here is SDS-PAGE is run under non-reducing condition. **c.** Circular dichroism traces of *TRACeR* molecules. The proteins have been purified and tested > 3 times in this study with one representing data shown.

Notably, all *TRACeR* constructs are purified on SEC as with elution volume corresponding to dimer size (~30kDa), however, SDS-PAGE gels show a band according to monomeric binder size (~15kDa). This result suggests *TRACeRs* form non-covalently linked, domain-swapped dimer constructs, which is further validated in structural study.



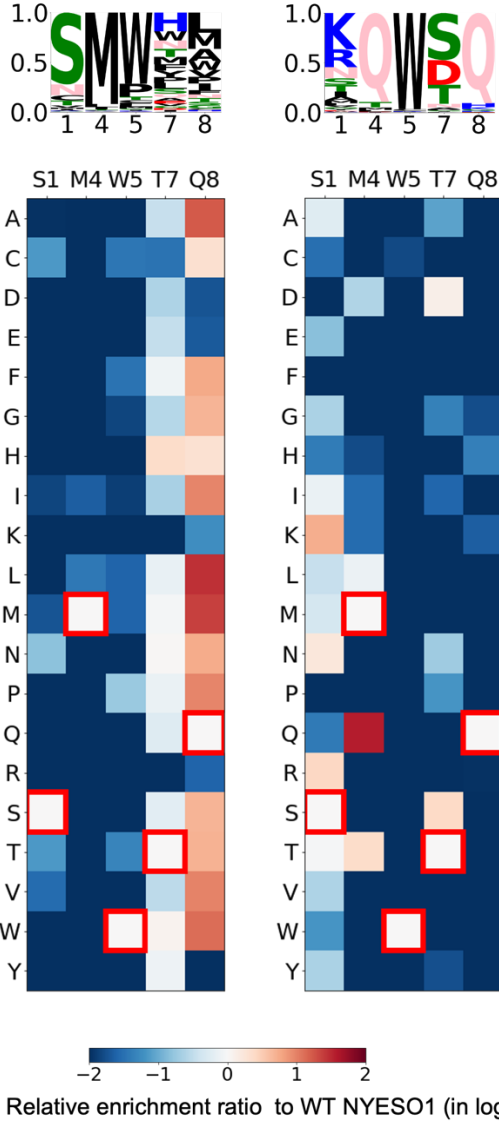
Supplementary Figure S8. Initial assessment of *TRACeR* specificity is performed by binding test with a random peptide library consisted of 50 peptides from TCGA library

Although performing thoroughly test with all possible peptides is impractical, this unbiased initial evaluation provides a clue on *TRACeR* has a low chance to cross-react with a random target. *TRACeR* was expressed on yeast surface and stained with anti-myc antibody with FITC fluorophore, and MHC tetramers with PE fluorophore at 50 nM tetramer concentration. Median Fluorescence Intensity (MFI) ratio is calculated based on $\frac{\text{MFI of FITC+ population}}{\text{MFI of FITC- population}}$

a**b**

Supplementary Figure S9. Staining and selection process on the triplet scanning library

a. Titration of biotinylated *TRACeR* binding on yeast expressing HLA-A*02:01 with NY-ESO-1 peptides (either SLLMWITQC or SLLMWITQV) and the negative control MART-1 peptide (ELAGIGILTV). Peptide-MHCs were displayed as a single chain construct consisting of peptide, B2M, and the HLA-A*02:01 alpha chain ectodomain, separated by GS linkers. **b.** Representative FACS gates used to select for peptides with binding to (top) *TRACeR* and (bottom) 1G4 TCR in a yeast-displayed NY-ESO-1 triplet NNK scanning library. Binding to NY-ESO-1 and MART-1 peptides in HLA-A*02:01 are shown as positive and negative controls, respectively. An EC₈₀ concentration of 156 nM *TRACeR*, derived from on-yeast titrations (a), was used to stain yeast libraries for selections. 1G4 TCR was stained using 100 nM tetramer conditions given a lack of detectable monovalent binding affinity on yeast. Peptide variants were enriched over a course of three rounds of FACS selections.



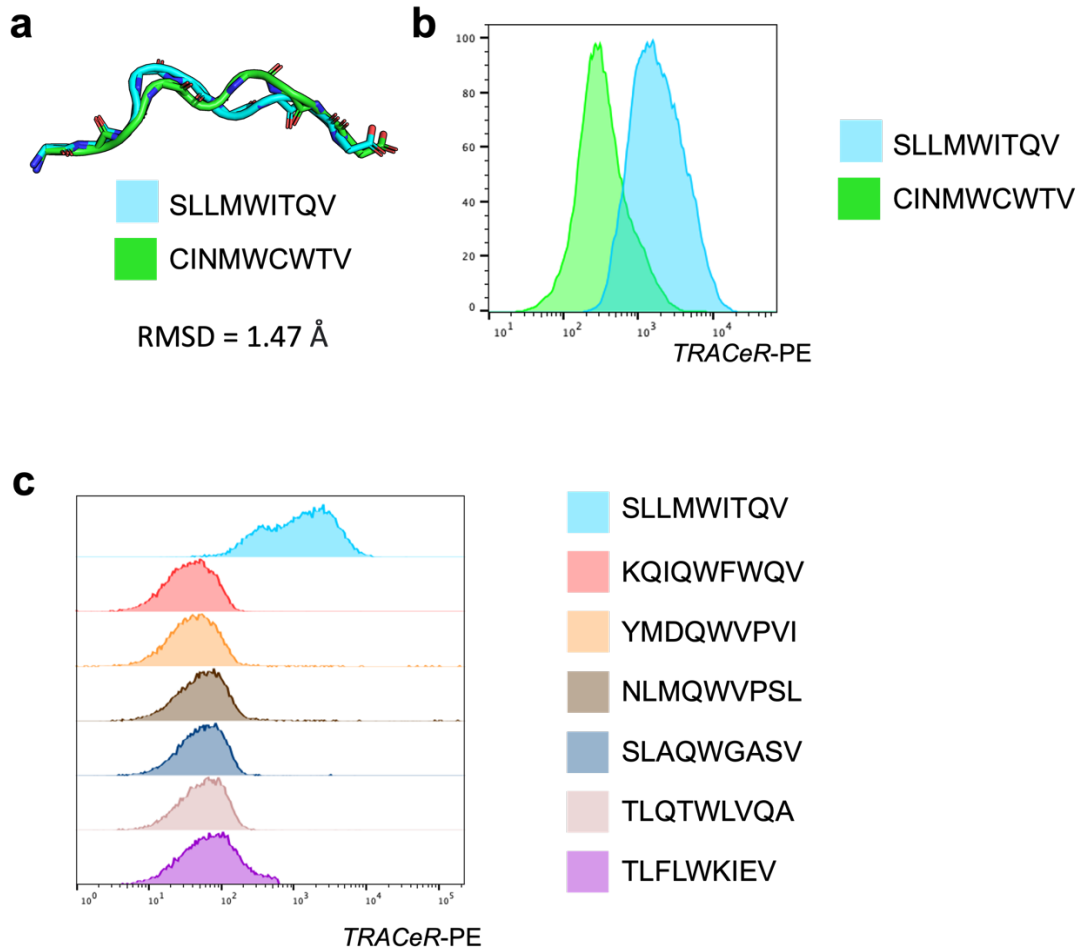
Supplementary Figure S10. SSM analysis on the triplet scanning library

Analysis was performed with treating mutations on each position as independent events. Data was analyzed using python by calculating the relative enrichment of each amino acid identity at each peptide position.

$$Enrichment_{x,i} = \frac{Final_pool_count_{x,i}}{Naive_pool_count_{x,i}}$$

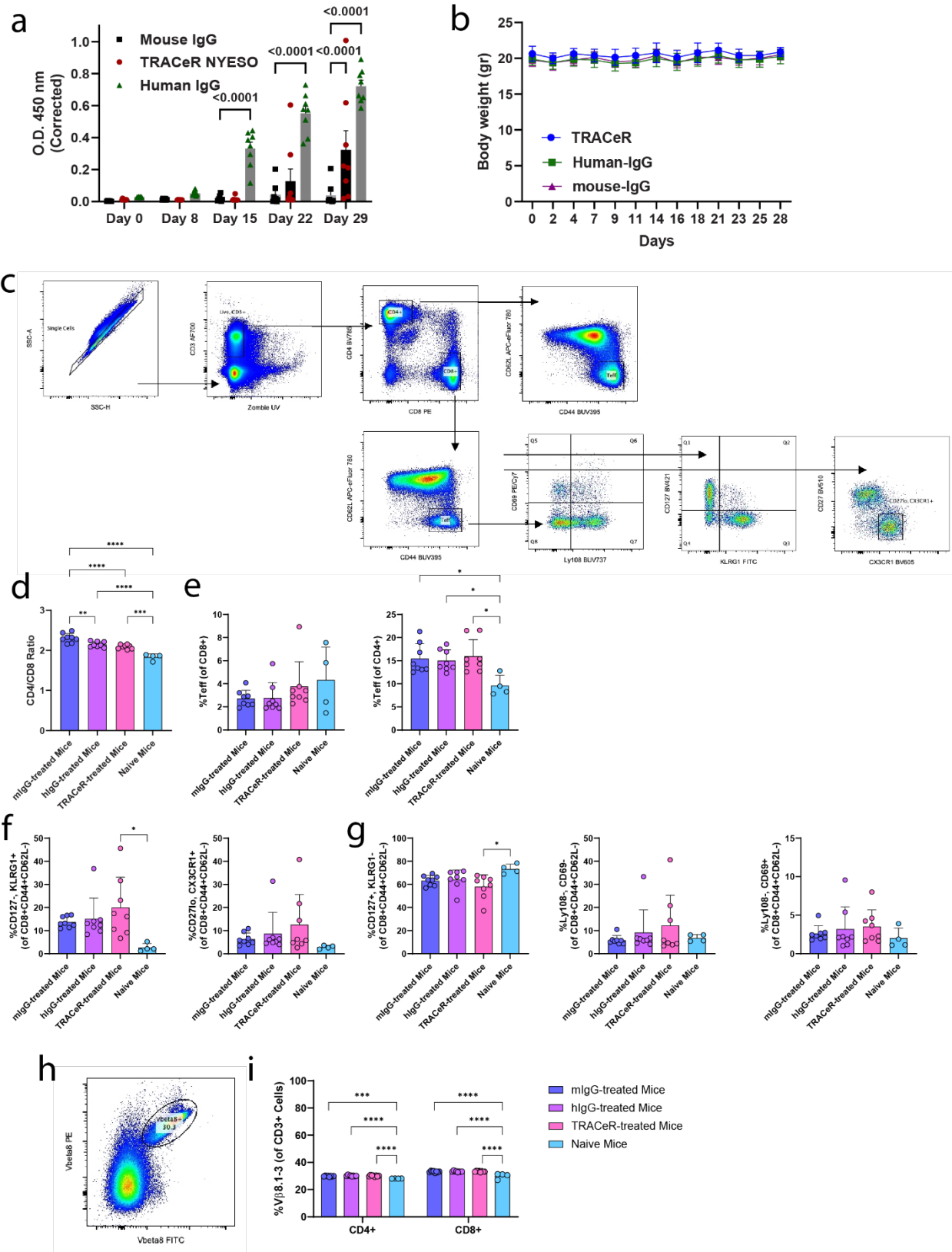
$$Relative_enrichment_{x,i} = \log_{10}\left(\frac{Enrichment_{x,i}}{Enrichment_{WT,i}}\right)$$

Blue denotes depletion relative to the WT, red denotes enrichment relative to the WT. The sequence logo was plotted based on the normalized enrichment ratio on each amino acid per position.



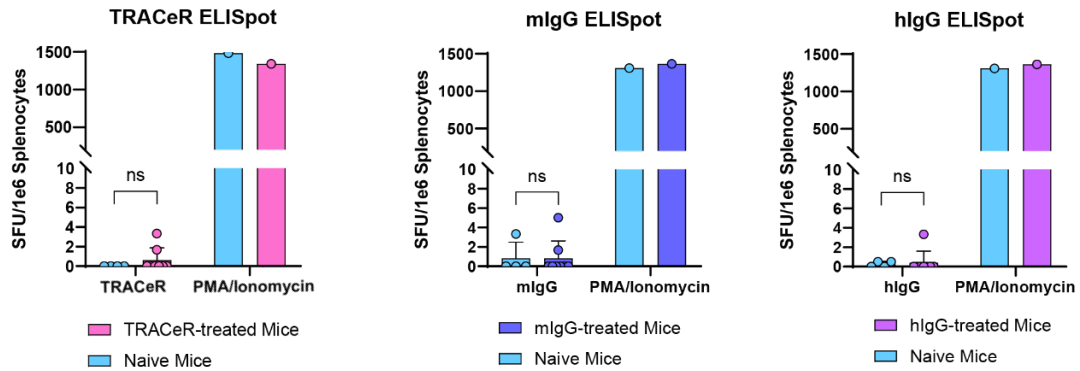
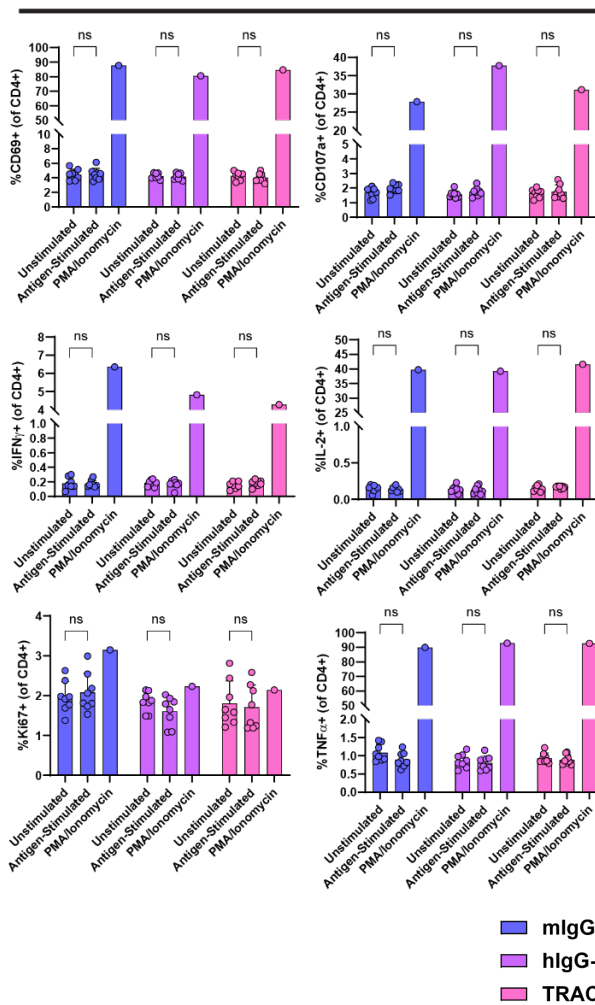
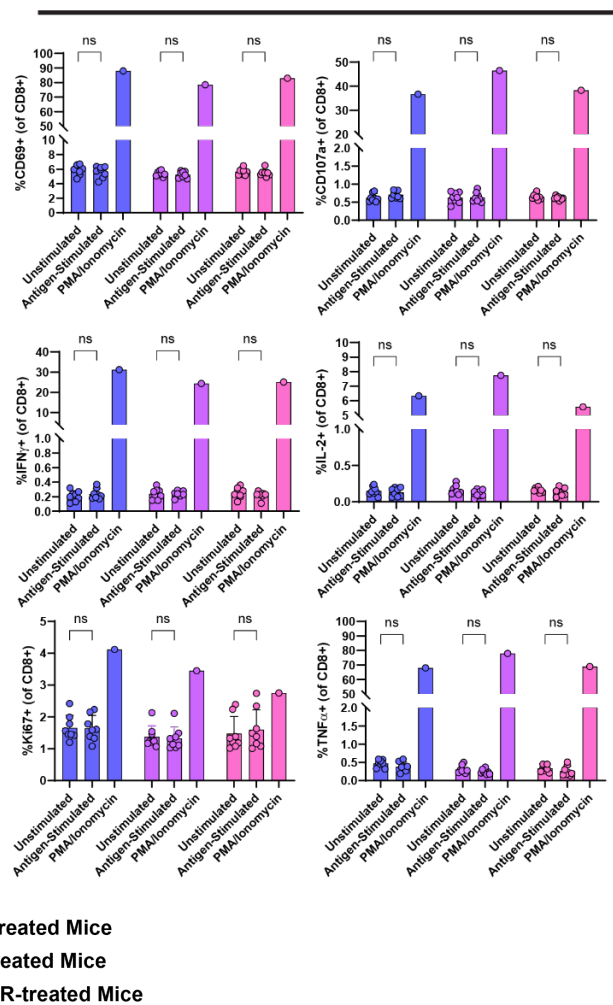
Supplementary Figure S11. Peptide selection of $TRACeR^{NY-ESO-1}_{MHC-I,A02}$

a. Backbone conformation comparison of NY-ESO-1 peptide SLLMWITQV (cyan) and CINMWCWTV (green). **b.** $TRACeR^{NY-ESO-1}_{MHC-I,A02}$ can differentiate SLLMWITQV and CINMWCWTV peptides expressed on HLA-A*02:01. $TRACeR^{NY-ESO-1}_{MHC-I,A02}$ was expressed on yeast and stained with 25 nM tetramer for each peptide. The CINMWCWTV peptide was identified based on sequence-based similarity searching from IEDB. We searched the IEDB for linear peptides which were positive for binding to HLA-A*02:01 (72,900 peptides), with the SMW motif using the following regular expression: “[SCT]..MW”. **c.** $TRACeR^{NY-ESO-1}_{MHC-I,A02}$ can differentiate SLLMWITQV and possible human epitopes selected from HLA ligand ATLAS expressed on HLA-A*02:01. $TRACeR^{NY-ESO-1}_{MHC-I,A02}$ was expressed on yeast and stained with 25 nM tetramer for each peptide. FITC⁺ population for singlet yeast cells is gated and shown in the flow plot.



Supplementary Figure S12. *In vivo* and *ex vivo* evaluation of immunogenicity of $TRACeR_{MHC-I, A02}^{NY-ESO-1}$ (n=8 mice/group)

BALB/c mice (n=8 mice/group) were treated with 4 ug of *TRACeR*, human IgG or mouse IgG over the period of 4 weeks by intravenous retroorbital injection (total of 13 doses over 4 weeks). Serum was collected once per week and mice were weighed over the course of the experiment. Following 6 weeks rest, mice were then sacrificed and splenocytes collected for analysis. A group of n=4 naïve BALB/c mice were euthanized, and their spleens analyzed alongside experimental mice as a negative control. **a.** IgG responses against treatment were measured by enzyme-linked immunosorbent assay (ELISA, 1:500 serum). Significance was determined by two-way ANOVA followed by Dunnett's multiple comparisons testing. n=8 mice per group, mean values are shown +/- S.E.M. **b.** Weight of mice over the course of treatment. n=8 mice per group, mean values are shown +/- S.E.M. **c.** Gating strategy for phenotypic analysis of CD4+ and CD8+ T cells from spleen. For (d-g), data are presented as mean values +/- standard deviation. Significance was determined by one-way ANOVA followed by Tukey's multiple comparisons testing. * p<0.05, ****p<0.0001. **d.** Ratio of CD4/CD8 T cells for each group. **e.** Quantification of frequency of CD44+CD62L- effector T cells among CD4+ and CD8+ T cells. **f.** CD127-KLRG1+ short-lived effectors and CD27lo CX3CR1+ T cells, and **g.** CD127+KLRG1- memory precursor effectors as well as quantification of Ly108-CD69- intermediate and Ly108-CD69+ terminally exhausted T cells. **h.** Representative flow plot of TCR Vβ8+ cell gating. **i.** Frequency of TCR Vβ8.1/8.2+ TCR Vβ8.3+ (pooled antibody stain for TCR Vβ8.1-3) cells among CD4+ and CD8+ T cells. Data are presented as mean values +/- standard deviation. Significance was determined by one-way ANOVA followed by Tukey's multiple comparisons testing. *** p<0.001, ****p<0.0001.

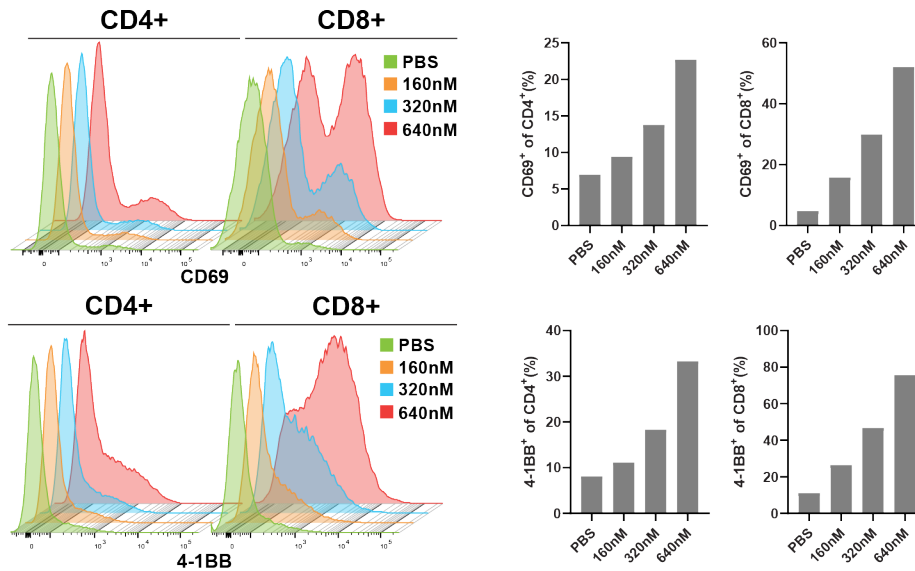
a**b****CD4****c****CD8**

Supplementary Figure S13. *TRACeR* do not produce significant long-term T cell responses and do not retain mitogenic activity. Splenocytes derived from treated mice from Supplementary Fig. S12 (n=8 mice/group) or naive mice (n=4) were restimulated with recombinant *TRACeR* or

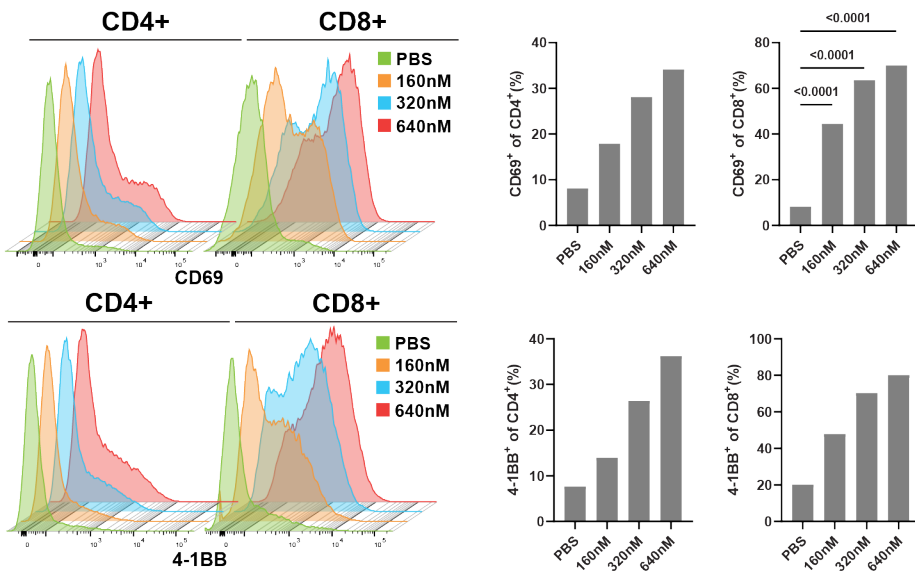
control IgG (mouse or human) for 16 hours (ELISpot) or 10 hours (intracellular cytokine staining). Splenocytes stimulated with PMA and ionomycin (n=1 from each group) were used as positive control for activation. **a.** The number of spot-forming units (SFU) per million splenocytes from each group following an IFN- γ ELISpot. Each biological replicate represents an average of two technical replicates for each condition. Data are presented as mean values $\pm$ standard deviation. Significance was determined by two-way ANOVA followed by Šídák's multiple comparisons testing. **b.** Analysis of frequency of CD69 $^{+}$, CD107 $^{+}$, IFN- γ $^{+}$, IL-2 $^{+}$, Ki67 $^{+}$, TNF α $^{+}$ cells among CD4 $^{+}$ T cells after restimulation. Data are presented as mean values $\pm$ standard deviation. Significance was determined by two-way ANOVA followed by Tukey's multiple comparisons testing. **c.** Same as in (b) but analyzing CD8 $^{+}$ cells.

A

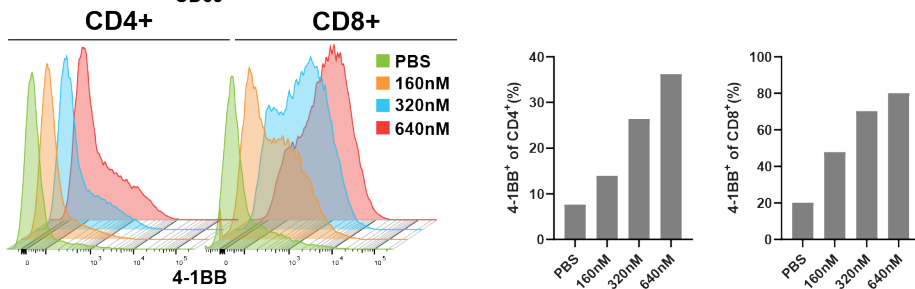
HBL-1



HLY-1

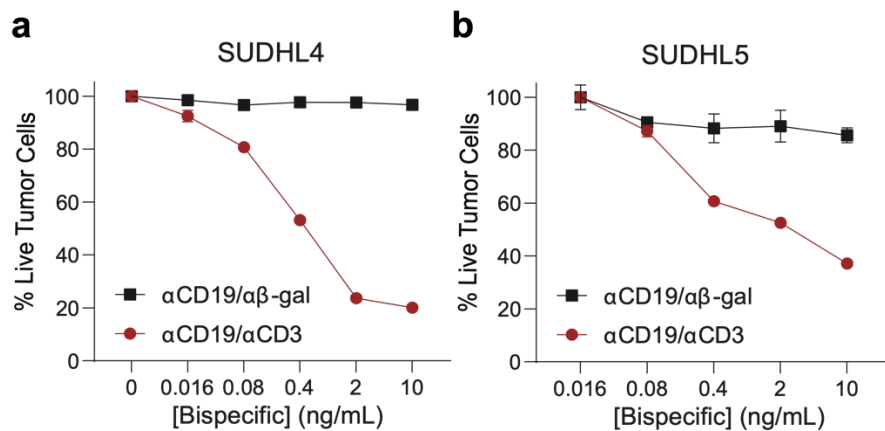


B

Supplementary Figure S14. Immune cell activation profile under *TRACeR* treatment

Anti-CD3/anti-CD28 activated total T cells were incubated with indicated tumor cell lines and treated with *TRACeR* BiTEs, control bispecific antibodies or PBS for 18 hours. Control bispecific antibodies were used at a concentration of 10 ng/mL. Cells were then isolated and analyzed by flow cytometry. Cells analyzed are live, CD3⁺CD4⁺ and CD3⁺CD8⁺ cells.

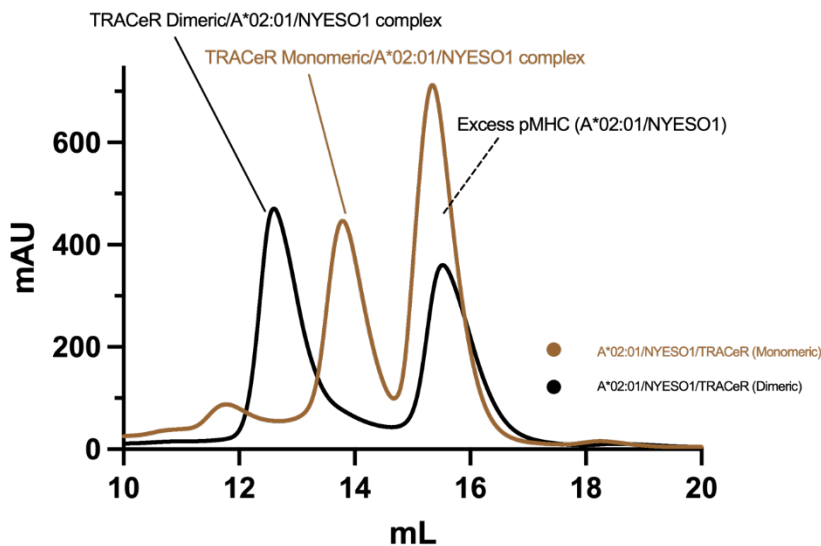
a. CD69 and 4-1BB expression in T cells incubated with HBL-1 cells. n=2 replicates per group. Mean is shown. **b.** CD69 and 4-1BB expression in T cells incubated with HLY-1 cells. n=2 replicates per group. Mean is shown.



Supplementary Figure S15. *In vitro* tumor cell killing assays with control BiTEs

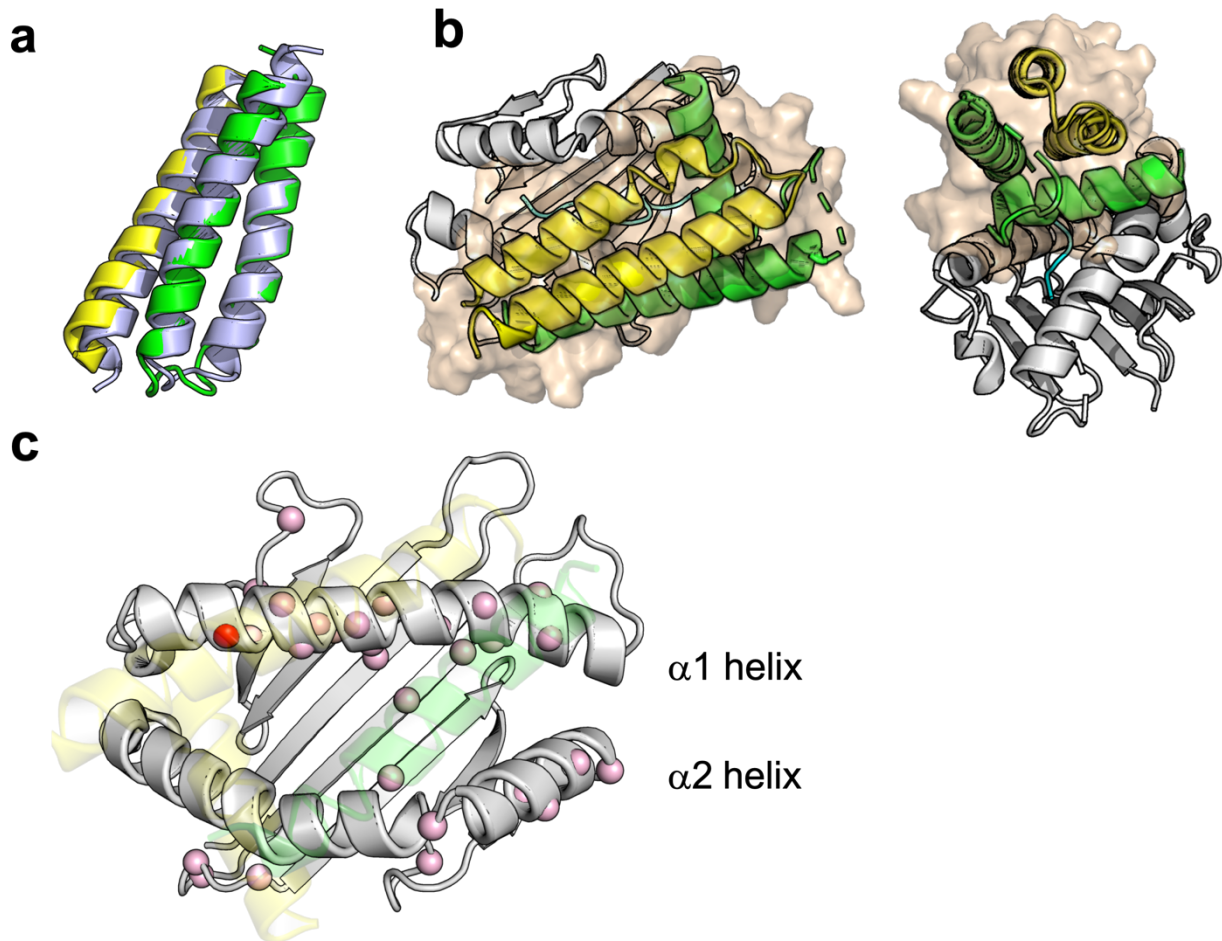
a. SUDHL4 cell (A02+, NY-ESO-1-) killing with antiCD19-antiCD3 control BiTEs. **b.** SUDHL5 cell (A02-, NY-ESO-1-) killing with antiCD19-antiCD3 control BiTEs.

Activated human total CD3⁺ cells were mixed 2:1 with tumor cell lines for 18 hours then analyzed for tumor specific killing by flow cytometry. Frequency of live tumor cells were normalized to PBS treated control cells. n=3 biological replicates is shown as mean +/- S.E.M.



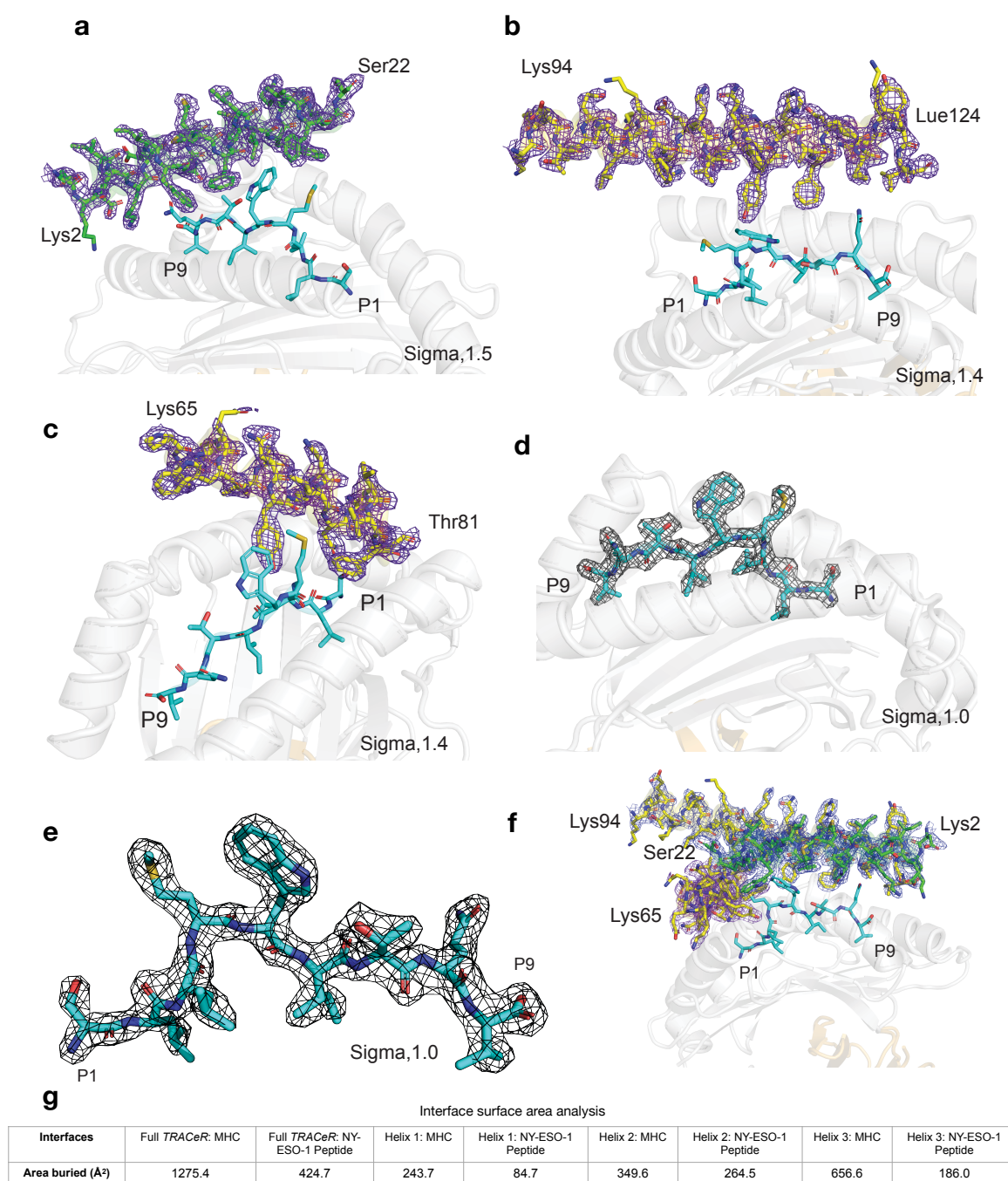
Supplementary Figure S16. SEC trace of the co-purification of the *TRACeR*^{NY-ESO-1}_{MHC-I A02} /HLA-A*02:01/NY-ESO-1 complex.

Dimeric *TRACeR*^{NY-ESO-1}_{MHC-I A02} was purified with HLA-A*02:01/NY-ESO-1 as a 2:2 complex, while monomeric *TRACeR*^{NY-ESO-1}_{MHC-I A02} was purified with HLA-A*02:01/NY-ESO-1 as a 1:1 complex.



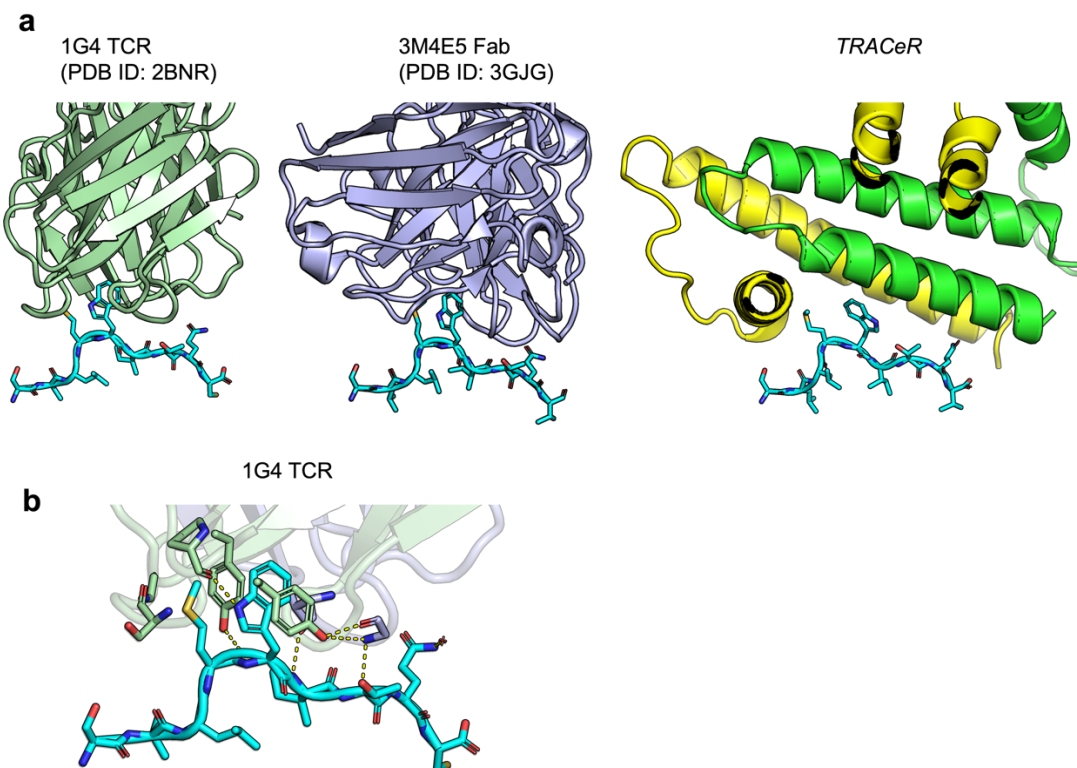
Supplementary Figure S17. Structure analysis of *TRACeR*^{NY-ESO-1}_{MHC-I A02}

a. Alignment of *TRACeR* helical bundle (yellow and green) with the helical bundle from MAM N terminal domain (grey). Backbone C α RMSD: 0.384 Å. **b.** Alignment of *TRACeR* monomeric binding mode with computational model. The computational displayed is the one with the lowest backbone RMSD from the crystal structure monomeric binding unit. (Model is shown in shadow). The models aligned head-to-tail in the context of the MHC but has the proper helical orientation. **c.** *TRACeR* on the MHC-I structure with spheres denoting positions where the sequences are different between A02 and B08.



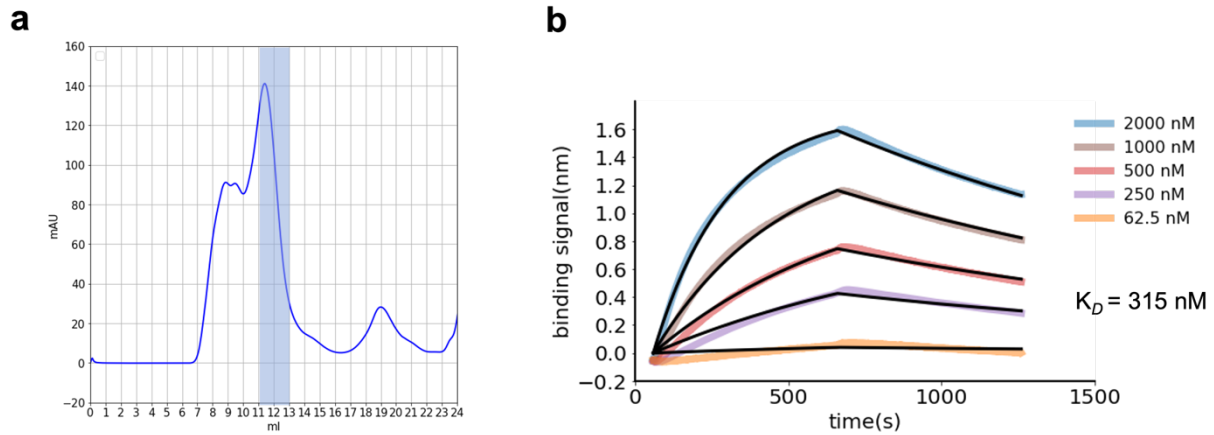
Supplementary Figure S18. Electron density omit maps shows interface of *TRACeR*-HLA-A*02:01/ β 2m/NY-ESO-1 complex. a. The 2Fo-Fc omit map around Helix 1 of *TRACeR* (molecule 1, green stick) contoured at 1.5 σ , shown in blue mash. **b & c.** 2Fo-Fc omit map around

Helix 4 and Helix 3 of *TRACeR* (molecule 2, yellow stick) contoured at $1.4\ \sigma$, shown in purple mesh. **d & e.** 2Fo-Fc omit map of NY-ESO-1 peptide (P1-P9, cyan stick) bound to A*02:01/ β 2m in *TRACeR*-A*02:01/ β 2m/NY-ESO-1 complex contoured at $1.4\ \sigma$, shown in black mesh. **f.** Overall view shows interaction of domain swapped helices (Helix 1, Helix 4 and Helix 3) of *TRACeR* to HLA-A*02:01/ β 2m/NY-ESO-1. **g.** Surface area analyses of *TRACeR* elements.



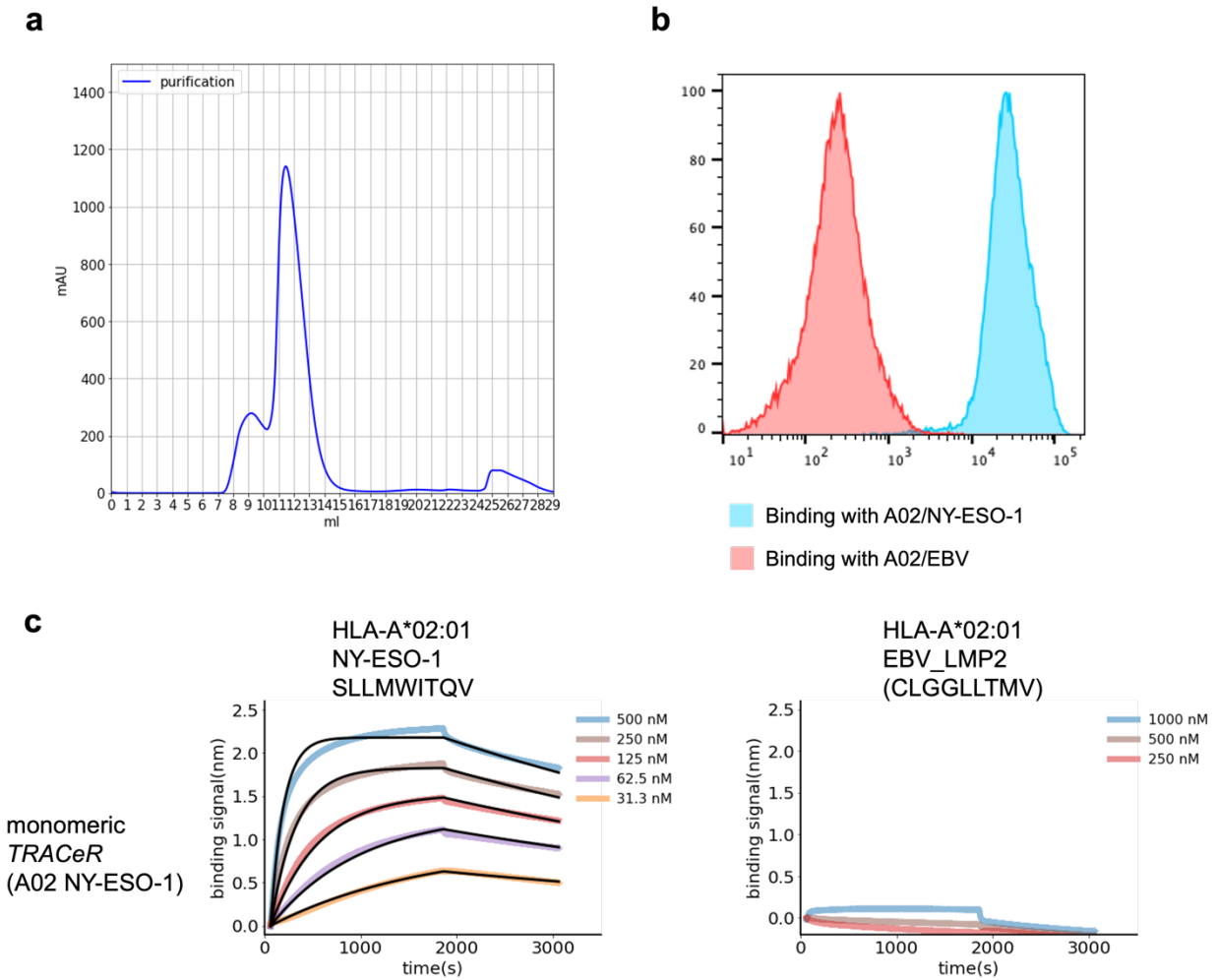
Supplementary Figure S19. Comparison of binding modes between TCR, Fab and *TRACeR* interacting with the same NY-ESO-1 peptide

a. Both TCR and Fab use 6 CDR loops to access the MHC-peptide interface. Due to the arcing conformation of MHC-I peptide, TCR and Fab are both not able to reach the first position on the peptide that is buried deep into MHC binding groove. The unique concave shape of *TRACeR* provides the ability to access and specifically recognize the first position. The *TRACeR* scaffold structurally also has the potential to access the Asn8 position. The current lack-of-ability of recognizing the Asn8 position is likely due to the current ARE selection strategy did not include optimization of Asn8 reaching positions on *TRACeR*. **b.** The interaction between 1G4 TCR and presented NY-ESO-1 peptide.



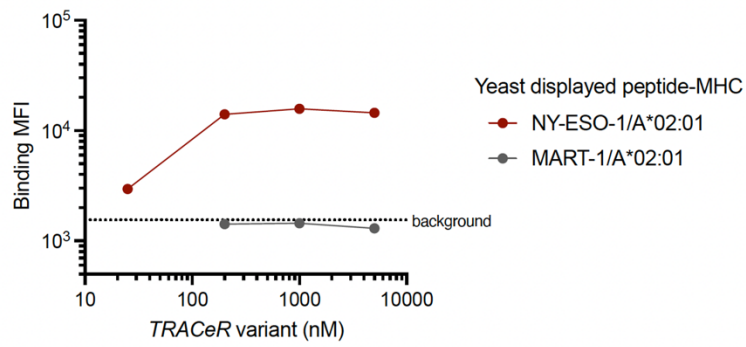
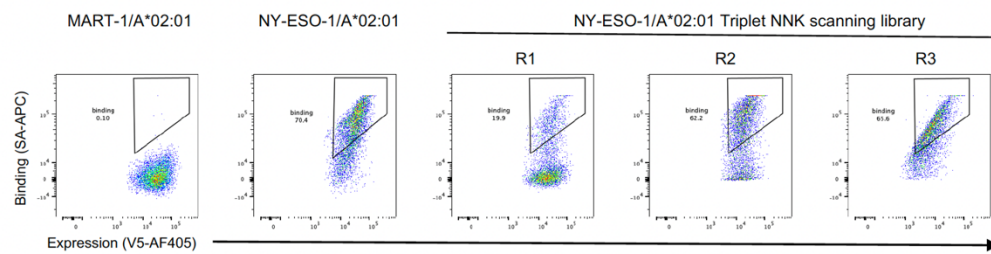
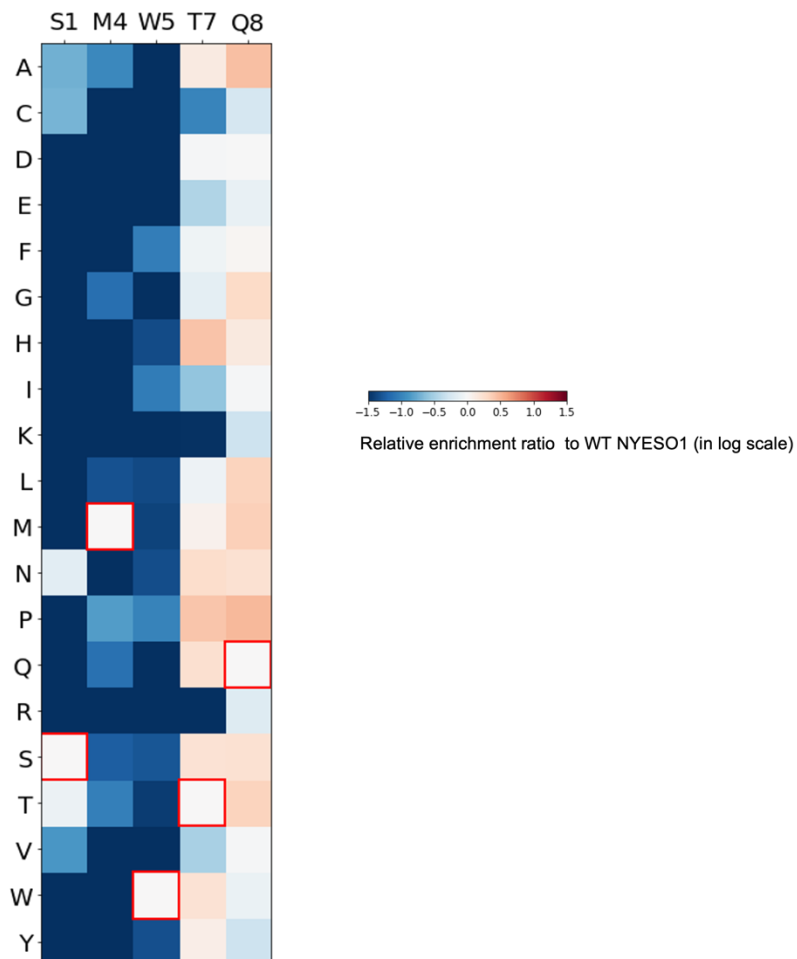
Supplementary Figure S20. Purification and affinity measurement of *TRACeR*^{NY-ESO-1}_{MHC-I,A02} with improved specificity

- a.** SEC plot of *TRACeR* constructs purification after purifying with Nickel column. Collected elution peak for downstream characterization and affinity determination is colored in shadow.
- b.** Affinity measurement of the specificity-improved *TRACeR* with Octet BLI.



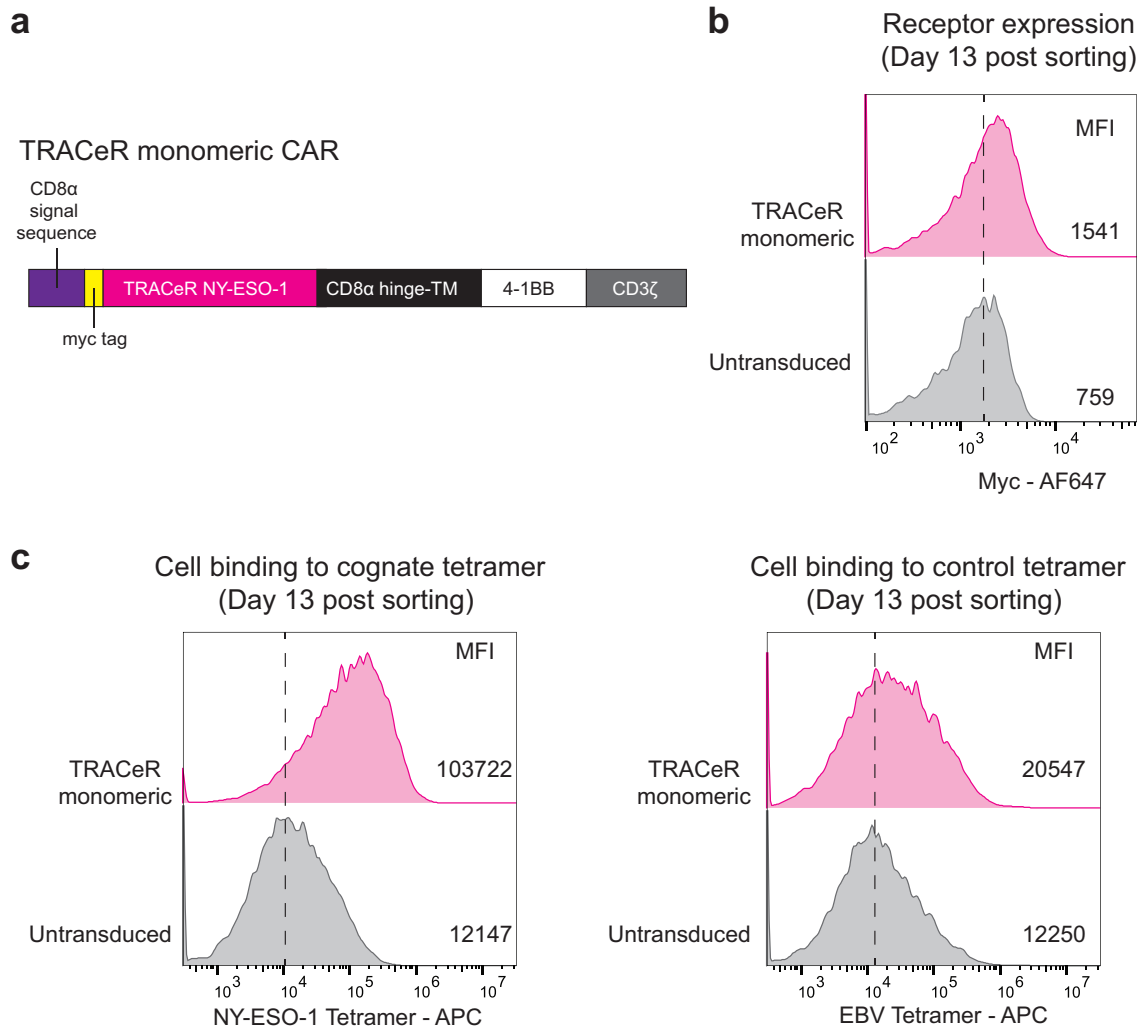
Supplementary Figure S21. Purification and binding specificity of rewired monomeric *TRACeR*

a. SEC plot of the monomeric *TRACeR* construct purified from E.coli BL21(DE3) strain **b.** monomeric *TRACeR*^{NY-ESO-1}_{MHC-I,A02} differentiates NY-ESO-1 peptide and EBV peptide presented on HLA-A*02:01. *TRACeR* was expressed on yeasted surface and stained with NY-ESO-1 and EBV tetramer at 50 nM concentration. **c.** Specificity characterization by BLI, with on- (NY-ESO-1) and off- (EBV) target pMHCs.

a**b****c**

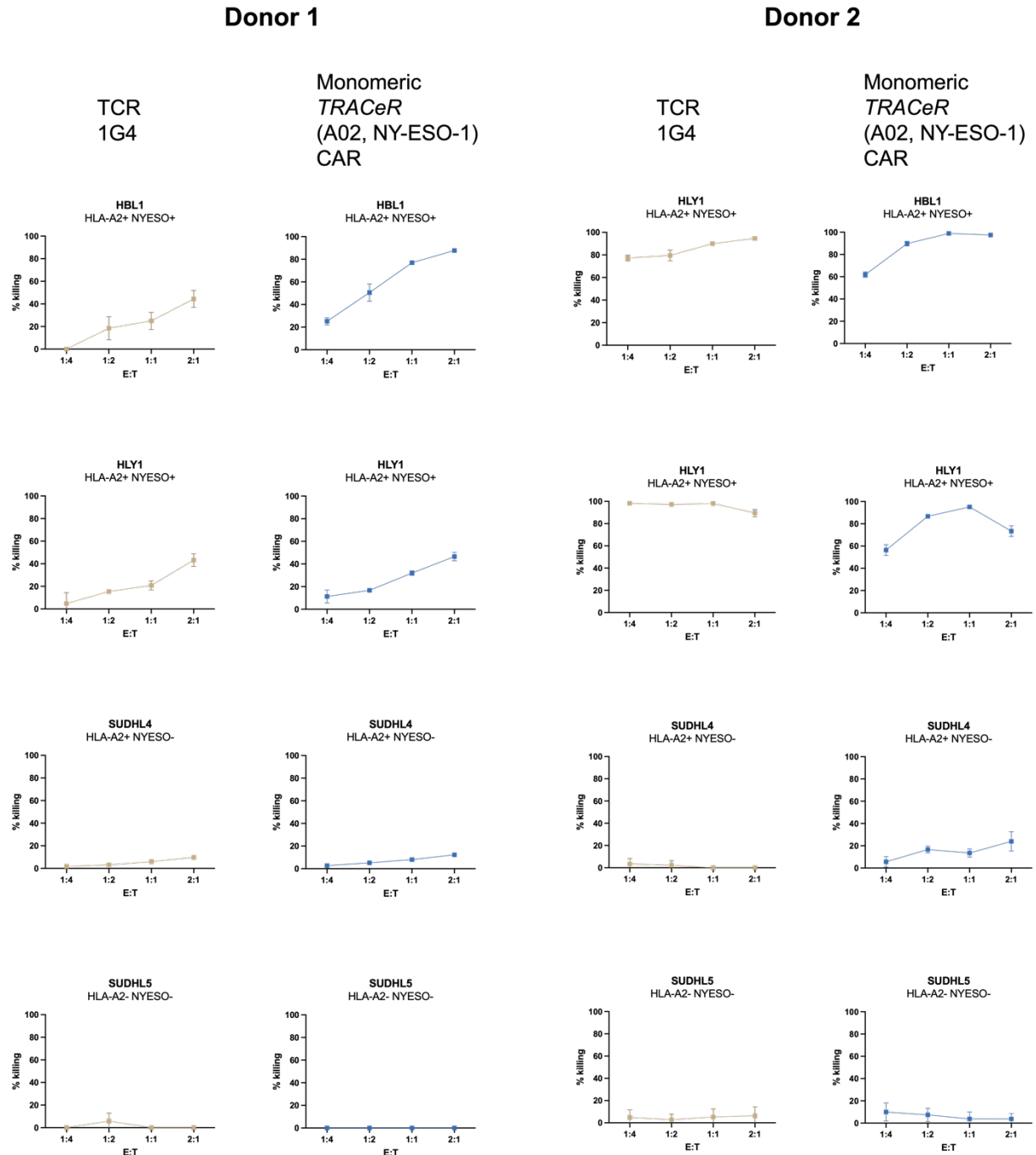
Supplementary Figure S22 Specificity of the rewired monomeric *TRACeR* determined by the yeast displayed triplet scanning library

a. Monomeric *TRACeR* variant binding to yeast expressing HLA-A*02:01 with NY-ESO-1 peptides and the negative control MART-1 peptide (ELAGIGILTV). Peptide-MHCs were displayed as a single chain construct consisting of peptide, B2M, and the HLA-A*02:01 alpha chain ectodomain, separated by GS linkers. **b.** Representative FACS sort plots of selections using a triplet NNK scanning library of the NY-ESO-1 peptide in HLA-A*02:01. The yeast library was stained with the monomeric *TRACeR* molecule at a 0.2 uM concentration, selected based on the titration results (a), and peptide variants that retain binding were enriched over a course of three rounds of FACS selections. Binding to NY-ESO-1 and MART-1 are shown as positive and negative controls, respectively. **c.** SSM analysis of the rewired monomeric *TRACeR*. Analysis was performed with treating mutations on each position as independent events. Data was analyzed using python by calculating the relative enrichment of each amino acid identity at each peptide position. SSM analysis of the original dimeric construct which the rewiring was based on is shown in Supplementary Fig. S10. The equation for calculating the enrichment ratio is shown under Supplementary Fig. S10



Supplementary Figure S23 Receptor design, expression and tetramer binding of engineered T cell receptors

a. Engineered CAR construct design. **b.** Engineered receptor expression 13 days post sorting. Quantification of median fluorescent intensity of anti-myc signal. **c.** Binding of engineered T cells to cognate HLA-A*02:01 human NY-ESO-1 157-165 C165V SLLMWITQV or control HLA-A*02:01 EBV LMP2 426-434 CLGGLTMTV tetramers. Quantification of median fluorescent intensity of each tetramer signal.



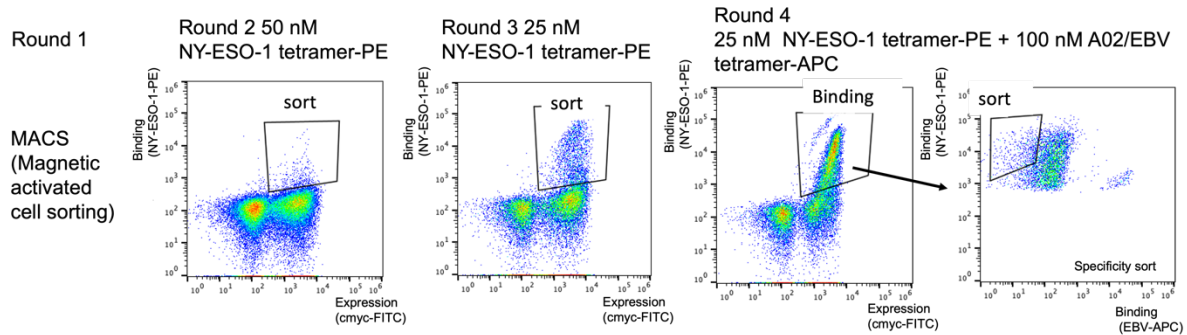
Supplementary Figure S24. In vitro tumor cell killing assays with *TRACeR*^{NY-ESO-1}_{MHC-I, A02}-CAR T cells in comparison with a CAR implemented with a TCR (1G4) as the binding domain.

In vitro tumor cell killing assays with *TRACeR*^{NY-ESO-1}_{MHC-I, A02}-CAR T cells derived from primary CD8⁺ T cells. Percentage of live tumor cells co-cultured with engineered T cells were normalized to those co-cultured with un-transduced T cells. n = 4 biological replicates for donor 1 and n=5 biological replicates for donor 2, shown as mean ± S.D.

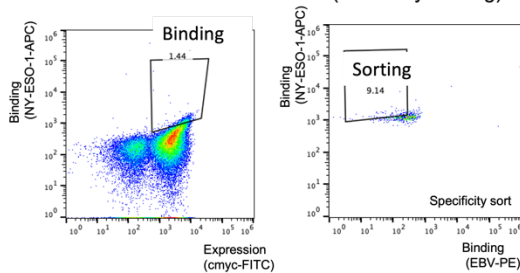
Master library sorting for 6 targets

Note : X nM tetramer staining means 4X nM pMHC monomer + avidity

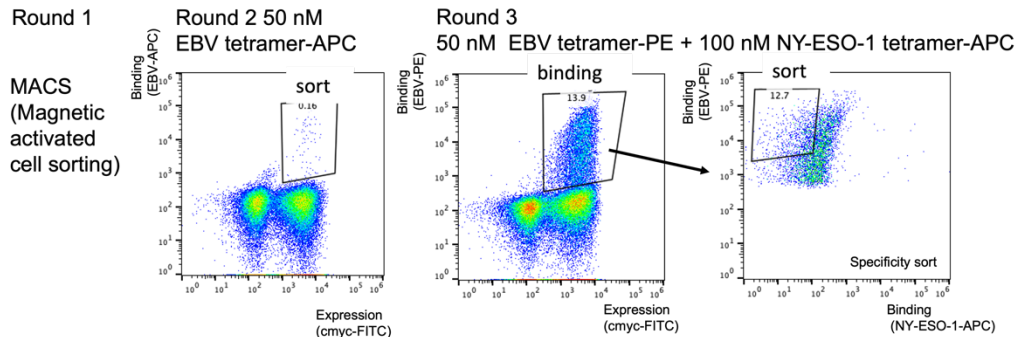
HLA-A*02:01, NY-ESO-1



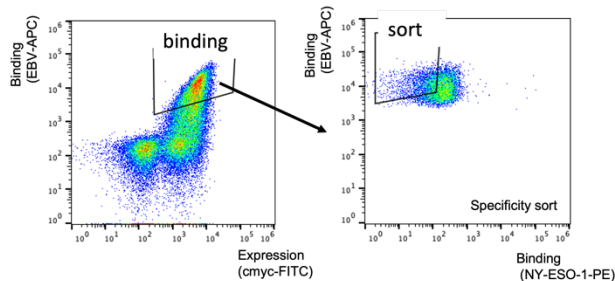
Round 5
100 nM NY-ESO-1 monomer-APC (no avidity binding) + 100 nM A02/EBV tetramer-PE



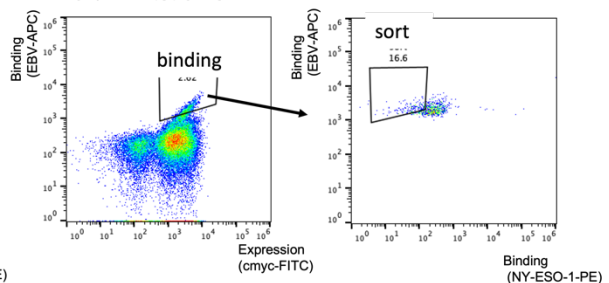
HLA-A*02:01, EBV



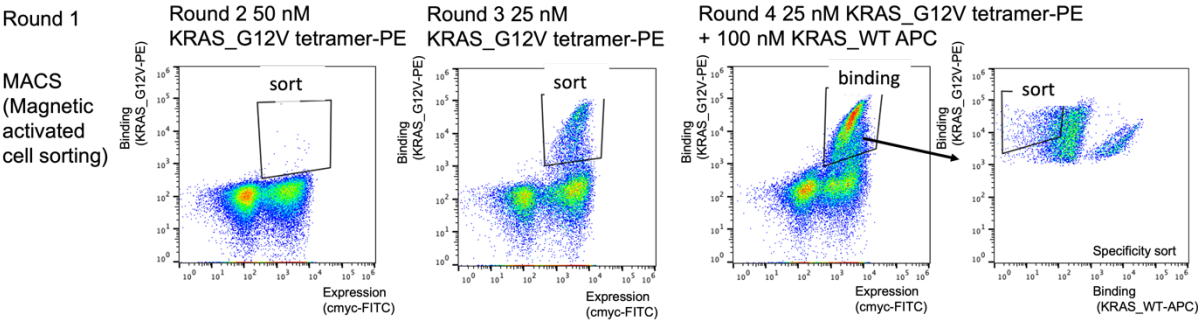
Round 4
25 nM EBV tetramer-APC + 100 nM NY-ESO-1 tetramer-PE



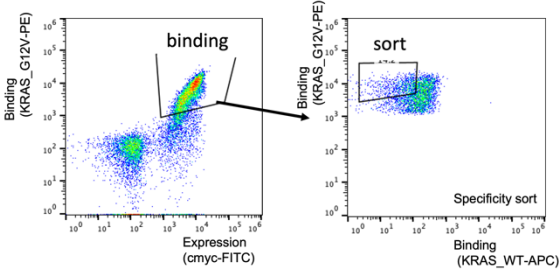
Round 5
100 nM EBV monomer-APC (no avidity binding) + 100 nM A02/EBV tetramer-PE



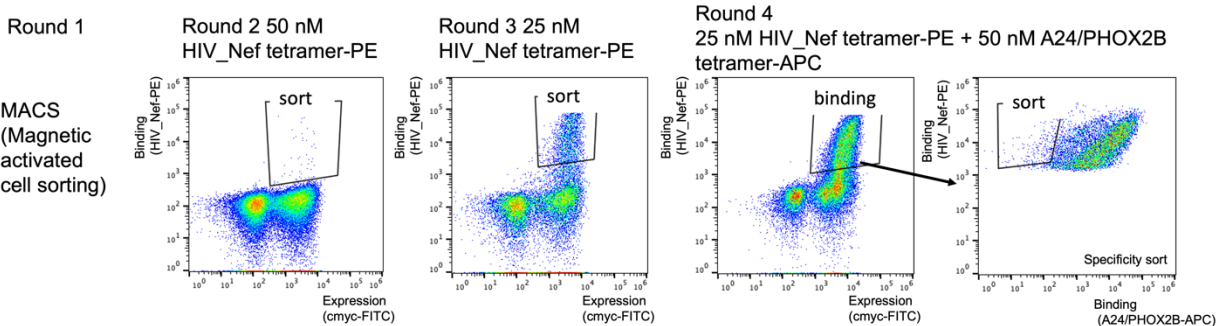
HLA-A*11:01, KRAS_G12V



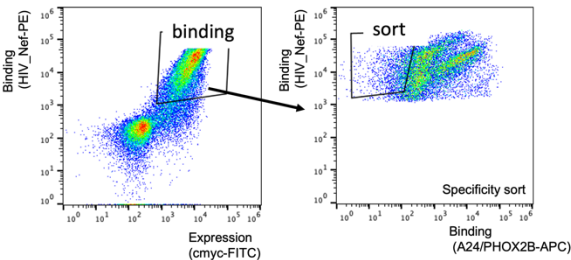
Round 5 5 nM KRAS_G12V tetramer-PE + 100 nM KRAS_WT APC



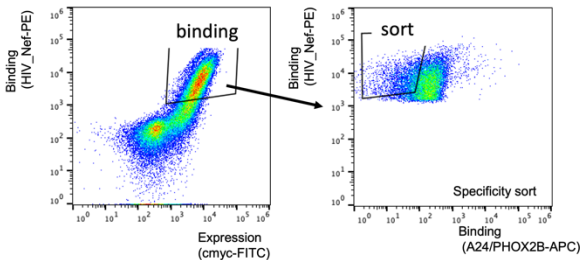
HLA-A*24:02, HIV_Nef



Round 5
25 nM HIV_Nef tetramer-PE +
100 nM A24/PHOX2B tetramer-APC



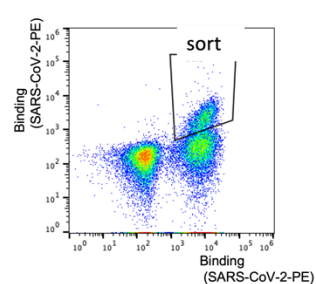
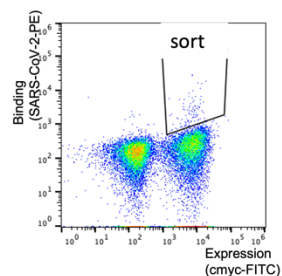
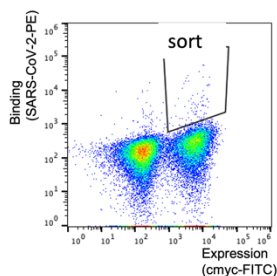
Round 6
10 nM HIV_Nef tetramer-PE +
100 nM A24/PHOX2B tetramer-APC



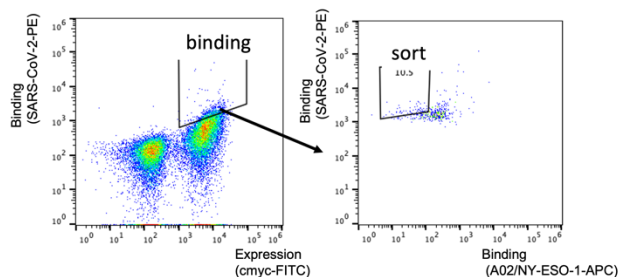
HLA-B*08:01, SARS-CoV-2

Round 1 Round 2 250 nM SARS-CoV-2 tetramer-PE Round 3 250 nM SARS-CoV-2 tetramer-PE Round 4 250 nM SARS-CoV-2 tetramer-PE

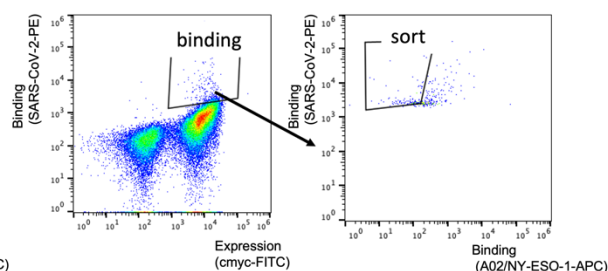
MACS
(Magnetic
activated
cell sorting)



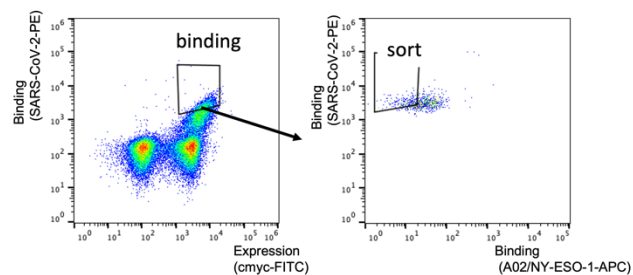
Round 5
125 nM SARS-CoV-2 tetramer-PE +
125 nM A02/NY-ESO-1 tetramer APC



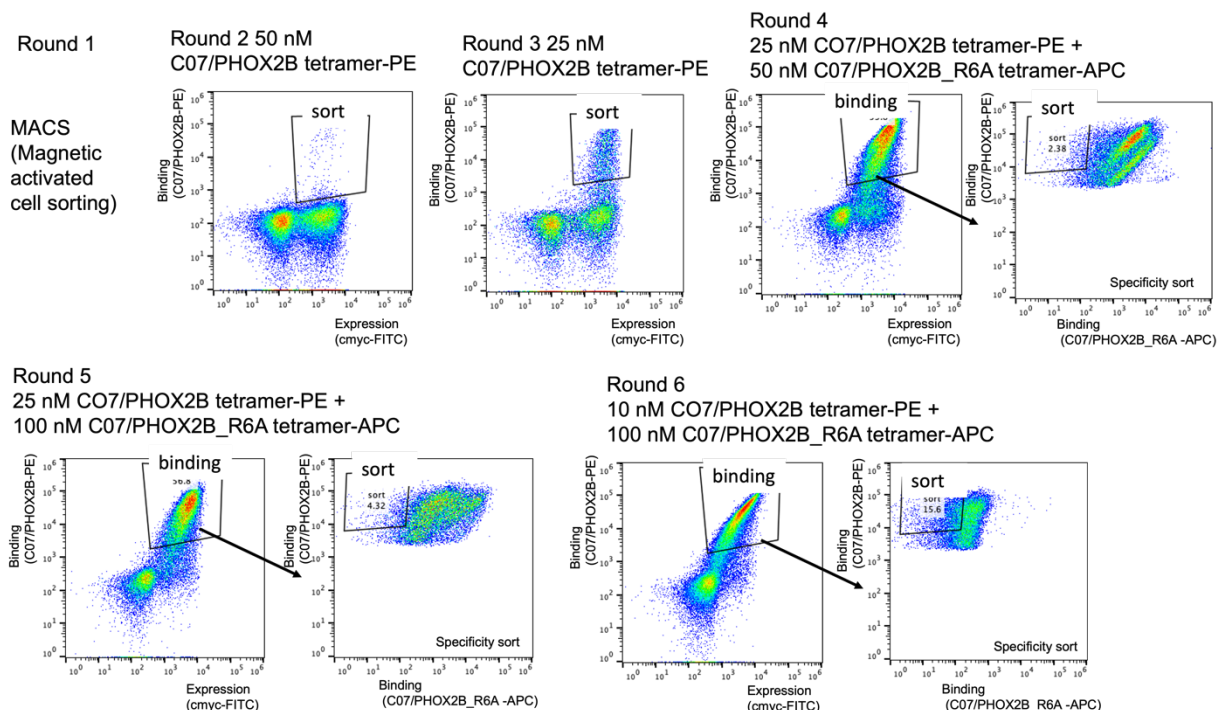
Round 6
62.5 nM SARS-CoV-2 tetramer-PE +
125 nM A02/NY-ESO-1 tetramer APC



Round 7
50 nM SARS-CoV-2 tetramer-PE +
125 nM A02/NY-ESO-1 tetramer APC



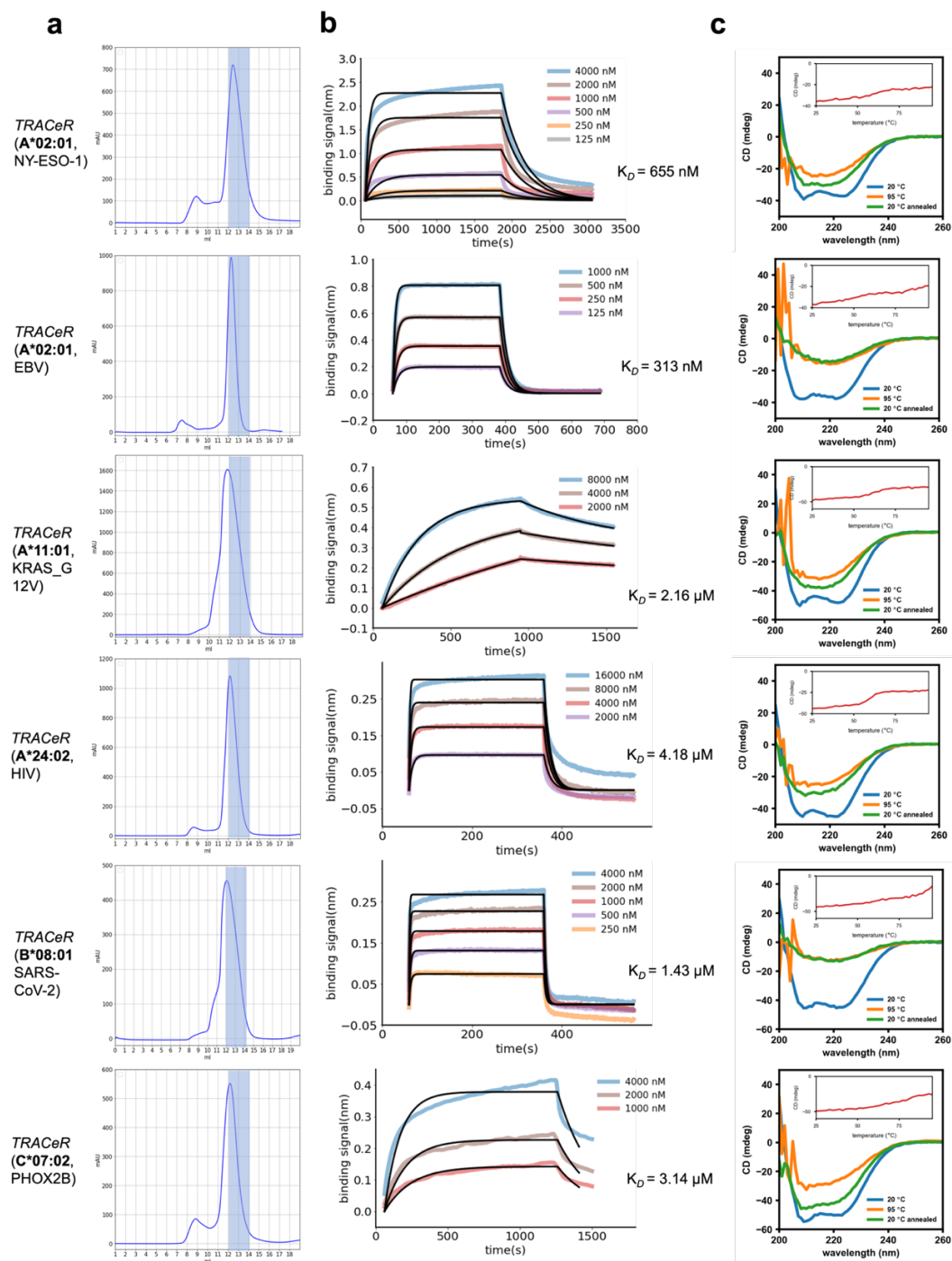
HLA-C*07:02, PHOX2B



Supplementary Figure S25. Enrichment process for all 6 targets from the master library

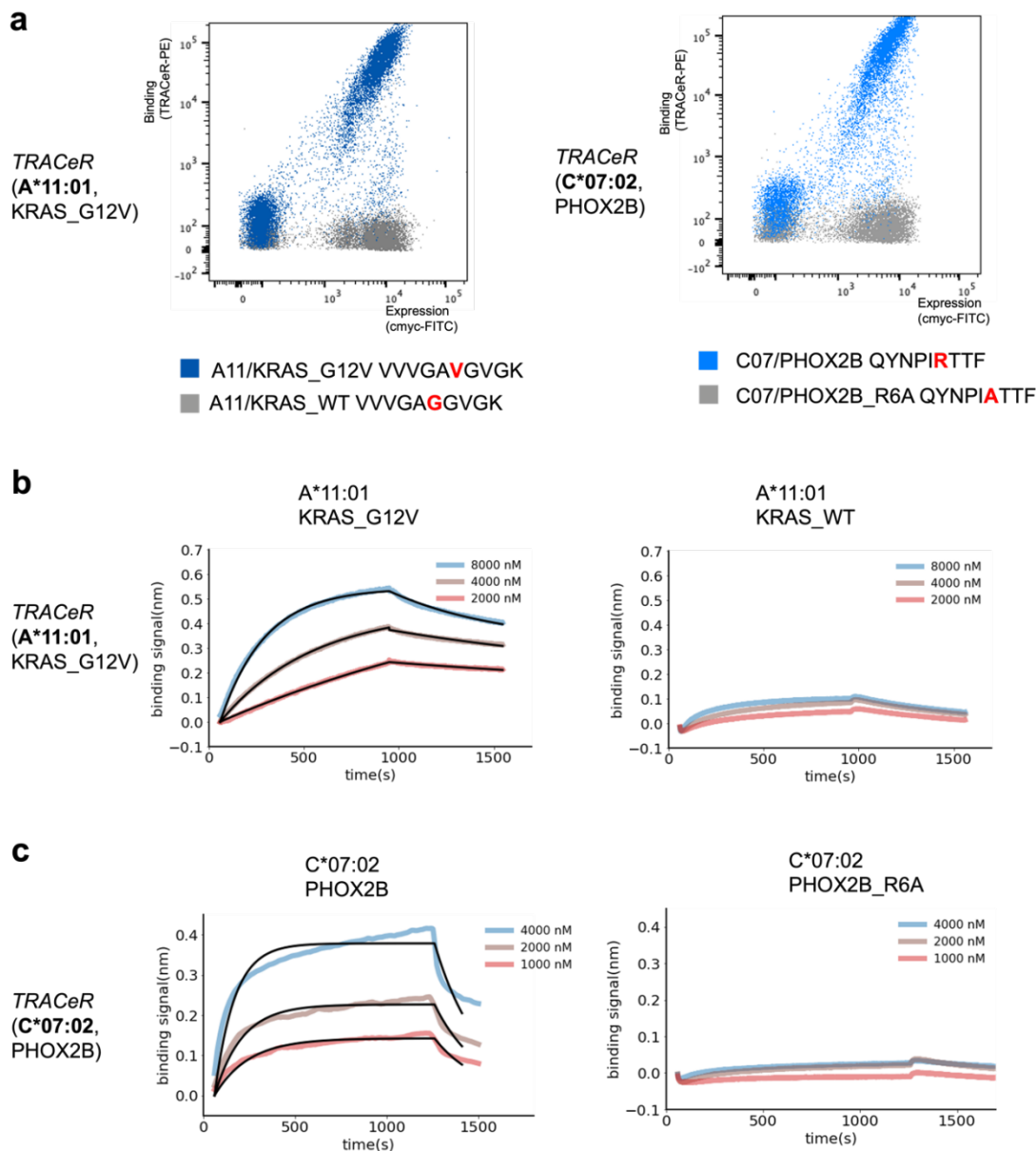
For each target, the labeling reagent and concentration for each round is noted in the figure. Different negative selection strategies were involved for different targets. A02/NY-ESO-1 *TRACeR* used A02/EBV tetramer as negative selection. A02/EBV *TRACeR* used A02/NY-ESO-1 tetramer as negative selection. A11/KRAS_G12V *TRACeR* used A11/KRAS_WT tetramer as negative selection. A24/Nef *TRACeR* used A24/PHOX2B tetramer as negative selection. B08/SARS-CoV-2 *TRACeR* used A02/NY-ESO-1 tetramer as negative selection. C07/PHOX2B *TRACeR* used C07/PHOX2B_R6A tetramer as negative selection.

The theoretical library size = $19^8 = 1.7 \times 10^{10}$ (8 randomized positions, each position has 19 amino acids without cysteine), For each target, 2×10^9 cells were processed during the first round of MACS.



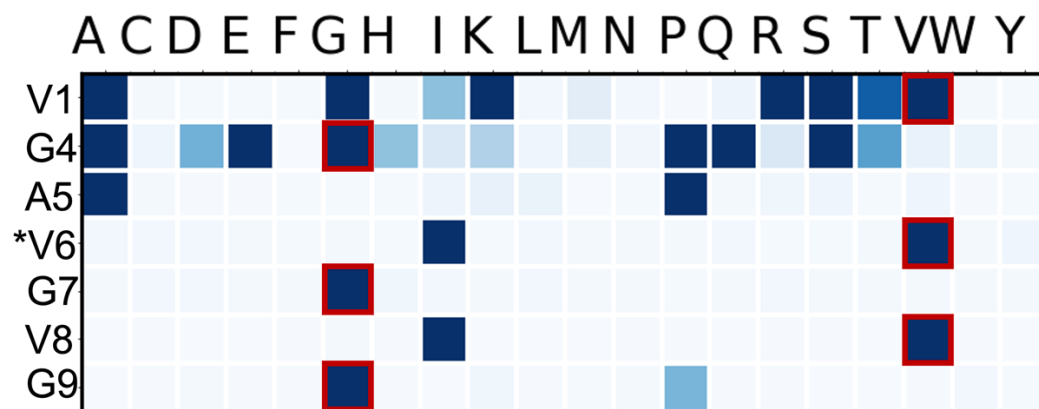
Supplementary Figure S26. Protein purification and characterization for *TRACeRs* selected from the monomeric master library

a. SEC plot of *TRACeR* constructs purification after purifying with Nickel column. Collected elution peak for downstream characterization and affinity determination is colored in shadow. **b.** Affinity measurement of *TRACeRs* with Octet BLI **c.** Circular dichroism traces of *TRACeR* molecules.



Supplementary Figure S27. *TRACeRs* selected from the master library can detect single amino acid difference on the peptide

a. Final round of *TRACeR* libraries were expressed on yeast surface and staining with according MHC tetramers at 50 nM tetramer concentration *TRACeR*^{KRAS_G12V}_{MHC-I,A11} differentiates KRAS_G12V and KRAS_WT at single G to V mutation. *TRACeR*^{PHOX2B}_{MHC-I,C07} differentiates PHOX2B and PHOX2B_R6A at single R to A mutation. **b.** Single residue specificity test with Octet BLI. *TRACeR*^{KRAS_G12V}_{MHC-I,A11} binding with KRAS_G12V and KRAS_WT. **c.** Single residue specificity test with Octet BLI. *TRACeR*^{PHOX2B}_{MHC-I,C07} binding with PHOX2B and PHOX2B_R6A.

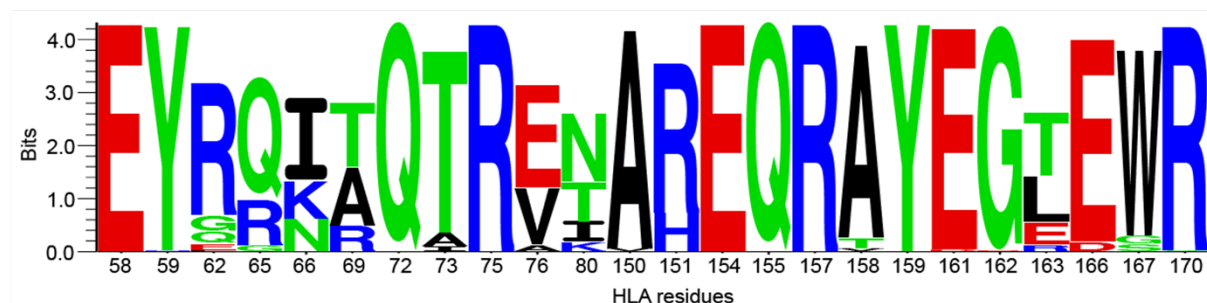


Supplementary Figure S28. SSM analysis of $TRACeR^{KRAS_G12V}_{MHC-I_A11}$ with tetramer staining

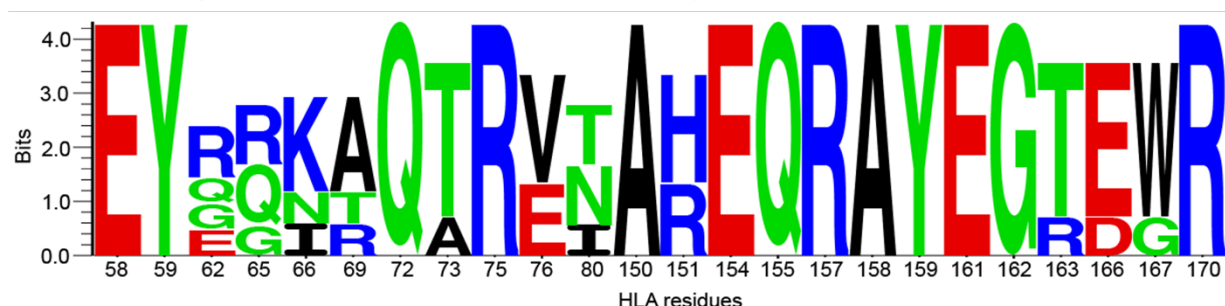
The staining was performed at 20 nM tetramer concentration (~the EC50 concentration of staining with the on-target KRAS_G12V). *V6 represent the G12V mutation position.

Positions of amino acids on MHC that have atom within 5 Å from *TRACeR* in the crystal structure: 58, 59, 62, 65, 66, 69, 72, 73, 75, 76, 80, 150, 151, 154, 155, 157, 158, 159, 161, 162, 163, 166, 167, 170, these positions are defined as “positions close to *TRACeR*”

Amino acid's identity for positions close to *TRACeR* on all common HLA alleles



Amino acid's identity for positions close to *TRACeR* among alleles that proven to be compatible with *TRACeR* (A*02:01, A*11:01, A*24:02, B*08:0, C*07:02)



Supplementary Figure S29. Comparison of sequence identities among all common alleles and alleles that are proven to be compatible with *TRACeR*

“Common” alleles were defined as those with a haploid frequency greater than 0.05% in at least one of the five broad race groups for a total of 215 HLA alleles across HLA-A, -B, and -C loci. HLA allele sequences were obtained from the IMGT/HLA database ¹

Tables

Supplementary Table 1. Summary of relevant amino acid sequences

Description	Amino acid sequence	Note
Library 1: Redesign MHC-II binder interface for binding MHC-I	MKLRCENPKKA??N?QNLNNVVFTNKELE EDIYDLSNKEETKEVLKLFKLKVNQFYRH AFGIVNDYGDKEIFNMMF??LW?VF?SQ??? ANNVE?IK?NI??LDWIMAEADNDLCYFIS Q	? Are library positions
Library 2: Create library on ARE for selecting binders for multiple targets	MKLRCENPK?????QNLNNVVFTNKELE DIYDLSNKEETKEVLKLFKLKVNQFYRHA FGIVNDYGDKEIFNMMFMMLWRVFRSQRID DANNVELIKFNIRVLDWIMAEADNDLCYFISQ	? Are library positions
TRACeR _{NY-ESO-1,A02} , from library 1 (initial scaffold)	MKLRCENPKKAYRDNAQNLNNVVFTNKELE EDIYDLSNKEETKEVLKLFKLKVNQFYRHAFGIVNDYGDKEIFNM MFMLLWRVFRSQRIDANNVELIKFNIRVLDWIMAEADNDLCYFISQ	
TRACeR _{NY-ESO-1,A02} , from library 2	MKLRCENPKEQWVANYQNLNNVVFTNKELE EDIYDLSNKEETKEVLKKFKEKVNQFYRHAFGIVNDYGDKEIFNM MFMLLWRVFRSQRIDANNVELIKFNIRVLDWIMAEADNDLCYFISQ	
TRACeR _{NY-ESO-1,A02} , Circular Permuted (Dimeric)	DKEIAKEIFNMMFMMLWRVFRSQRIDANNVELIKFNIRV LDWIMAEADNDLCYFIGTHDKCENPKEQWVANYQNL NNVVFTNKELE EDIYDLSNKEETKEVLKKFKEKVNQFYRHAFDIINKYG	
TRACeR _{EBV,A02} , Circular Permuted (Dimeric)	DKEIAKEIFNMMFMMLWRVFRSQRIDANNVELIKFNIRV LDWIMAEADNDLCYFIGTHDKCENPKNLALVFSQNLN NFVVFTNKELE EDIYDLSNKEETKEVLKKFKEKVNQFYRHAFDIINKYG	
TRACeR _{NY-ESO-1,A02} -antiCD3	MDIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQ QKPGKAPKLLIYYTSRLESGVPSRFSGSGSGTDYTLTISS LQPEDFATYYCQQGNTLPWTFGQGTKVEIKGGGSGGG SGGGSGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAAS GYSFTGYTMNWVRQAPGKGLEWVALINPYKGVSTYN QKFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYYCAR SGYYGDSDWYFDVWGQGTLLTVSGGGGSLEVLFQGP DKEIAKEIFNMMFMMLWRVFRSQRIDANNVELIKFNIRV LDWIMAEADNDLCYFIGTHDKCENPKEQWVANYQNL	

	NNVVFTNKELEDIYDLSNKEETKEVLKKFKEKVNQFYR HAFDIINKYGGGHHHHHH
TRACeR _{NY-ESO-1} , monomer	SVEEIKKEYEERLKRFDDEFVERILKETGNKEIANMARM LLWRVERSYRIDKDNVELIKFNIRVIDWIMAEAEENDLCYF IGTHDKCENPKEQWVANYQNLNNVVFTNKELEDIYDE SNKEETKEVLKKFKEKVNQFYEHAFDIINKYGDKEIFN MMFMLLWRVFRSFRIDANNVELIKFNIRVLDWIMAEAD NDLSYFISQ
TRACeR CAR design	MALPVTALLPLALLHAARPS SVEEIKKEYEERLKRFDDEF VERILKETGNKEIANMARMLLWRVERSYRIDKDN VELIKFNIRVIDWIMAEAEENDLCYFIGTHDKCENPK EQWVANYQNLNNVVFTNKELEDIYDESNKEETKE VLKKFKEKVNQFYEHAFDIINKYGDKEIFNMMFM LLWRVFRSFRIDANNVELIKFNIRVLDWIMAEADN DLSYFISQTTTPAPRPPTPAPTIASQPLSLRPEACRPA AGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVI TLYCK RGRKKLLYIFKQPFMRPVQTTQEEDGCS CRFP EEEEGGCELRVKFSRSADAPAYKQGQNQLYNEL NLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEG LYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQ GLSTATKDTYDALHMQALPPR* <i>CD8 Signal peptide -</i> HLA-A2/NYESOv TRACeR CD8 Transmembrane 4-1BB Signaling Domain CD3 Zeta
TRACeR _{NY-ESO-1,A02} , monomeric (re-selected from the monomeric library)	SVEEIKKEYEERLKRFDDEFVERILKETGNKEIANMARM LLWRVERSYRIDKDNVELIKFNIRVIDWIMAEAEENDLCYF IGTHDKCENPKEQWVANYQNLNNVVFTNKELEDIYDE SNKEETKEVLKKFKEKVNQFQEHAFDIINKYGDKEIMN MMQMLLWRVFRSFRIDANNVELIKFNIRVLDWIMAEA DNDLSYFISQ
TRACeR _{EBV,A02} , monomeric	SVEEIKKEYEERLKRFDDEFVERILKETGNKEIANMARM LLWRVERSYRIDKDNVELIKFNIRVIDWIMAEAEENDLCYF IGTHDKCENPKEQWVANEQNLWNVVFTNKELEDIYDE SNKEETKEVLKKFKEKVNQFEEHAFDIINKYGDKEITN MMYMLLVRVHRSFRIDANNVELIKFNIRVLDWIMAEA DNDLSYFISQ
TRACeR _{KRAS_G12V,A11} , monomeric	SVEEIKKEYEERLKRFDDEFVERILKETGNKEIANMARM LLWRVERSYRIDKDNVELIKFNIRVIDWIMAEAEENDLCYF IGTHDKCENPKEQWVANHQNLNDVVSTNKELEDIYDE SNKEETKEVLKKFKEKVNQFREHAFDIINKYGDKEITN

	MMMMLLFRVQRSFRIDANNVELIKFNIRVLDWIMAEA DNDLSYFISQ
TRACeR _{Nef_HIV,A24} , monomeric	SVEEIKKEYEERLKRFDDEFVERILKETGNKEIANMARM LWRVERSYRIDKDNVELIKFNIRVIDWIMAEAENDLCYF IGTHDKCENPKEQWVANWQNLTNVVNTNKELEDIYDE SNKEETKEVLKKFKEKVNQFNEHAFDIINKYGDKEIWN MMGMLLVRVTRSFRIDANNVELIKFNIRVLDWIMAEAD NDLSYFISQ
TRACeR _{SARS-CoV-2,B08} , monomeric	SVEEIKKEYEERLKRFDDEFVERILKETGNKEIANMARM LWRVERSYRIDKDNVELIKFNIRVIDWIMAEAENDLCYF IGTHDKCENPKEQWVANAQNLHNVVETNKELEDIYDE SNKEETKEVLKKFKEKVNQFWEHAFDIINKYGDKEIWN MMTMLLHRVNRSFRIDANNVELIKFNIRVLDWIMAEAD NDLSYFISQ
TRACeR _{PHOX2B,C07} , monomeric	SVEEIKKEYEERLKRFDDEFVERILKETGNKEIANMARM LWRVERSYRIDKDNVELIKFNIRVIDWIMAEAENDLCYF IGTHDKCENPKEQWVANEQNLNVVFTNKELEDIYDES NKEETKEVLKKFKEKVNQFWEHAFDIINKYGDKEIDNM MDMLLHRVDRSFRIDANNVELIKFNIRVLDWIMAEADN DLSYFISQ

Note: For the monomeric *TRACeR* design, we ran ProteinMPNN on non-interface surface residues to reduce the surface hydrophobicity.²

Supplementary Table 2. Binding kinetics summary of dimeric *TRACeRs*

TRACeR	K_D (M)	K_D error*	k_{on}	k_{on} error*	k_{dis}	k_{dis} error*
HLA-A02/ NY-ESO-1	2.17e-8	1.00e-9	9.77e2	1.86e0	2.12e-5	9.77e-7
HLA-A02/ EBV	9.36e-8	6.44e-9	4.91e2	1.29e1	4.6e-5	2.92e-6
HLA-B08/ SARS-CoV-2	3.71e-6	1.05e-7	3.34e2	8.89e0	1.24e-3	1.23e-5
HLA-A02/ NY-ESO-1 with specificity improved	3.15e-7	2.37e-9	1.83e3	1.06e1	5.76e-4	2.76e-6

* The errors are fitting errors of the kinetic binding measurements provided by the ForteBio Data Analysis software.

Supplementary Table 3. Predicted binding and thermal stabilities for exchanged HLA-A*02:01 bound to individual TCGA and NY-ESO-1 position 1, 4, 5, 7, and 8 full-scanning epitopes

Percent EL_{rank} is predicted by NetMHCpan – 4.1 compared to a set of random natural peptides eluted by mass spectrometry. T_m of individual peptides from the TCGA and NY-ESO-1 position 1, 4, 5, 7, and 8 full-scanning peptide libraries loaded on HLA-A*02:01 via peptide exchange were measured by DSF in triplicates. Refolded gTAX/HLA-A*02:01 and exchanged NY-ESO-1 (SLLMWITQV)/HLA-A*02:01 were used as controls. *S1V (VLLMWITQV) peptide failed to be synthesized due to its extreme hydrophobicity.

Peptide name	Sequence	EL _{Rank}	T _m Values (°C)			Averages	SD
NY-ESO-1	SLLMWITQV	0.0263	60.26	60.13	59.89	60.1	0.2
gTAX	LFGYPVYV	0.9486	41.92	41.97	42.20	42.03	0.1
P1	KLVVVGACGV	5.3829	50.27	48.72	48.64	49.21	0.9
P2	LLGRNSFEVHV	1.9134	45.86	42.73	42.62	44	2
P3	KLVVVGAAGV	4.2671	47.41	47.32	49.88	48	1
P4	RLIRVEGNLRV	0.1307	54.67	54.32	52.48	54	1
P5	KLVVVGASGV	4.3211	46.76	46.54	46.65	46.65	0.1
P6	ALNNMFCQLA	5.8956	43.73	46.09	44.89	45	1
P7	ILNREIDFA	1.6768	49.45	49.35	49.11	49.30	0.2
P8	KTPVQLWV	0.2649	50.10	50.03	49.89	50.01	0.1
P9	GLAPPQHRI	0.101	44.54	44.19	44.16	44.30	0.2
P10	YMFNSSCMGGM	5.6017	48.65	48.56	47.99	48.40	0.4
P11	LLGRNSFEMRV	3.029	47.69	47.74	47.65	47.69	0.0
P12	GLAPPQRLIRV	0.171	52.40	52.46	52.29	52.38	0.1
P13	YLSTDVGFCT	4.9509	49.04	49.09	48.82	48.98	0.1
P14	VLMGHVAAVG	2.7279	60.26	55.47	59.39	58	3
P15	YLDSGIHFG	1.4319	51.80	51.77	52.01	51.86	0.1
P16	KILNREIDFAI	3.4346	46.98	51.20	46.68	48	3
P17	YMCNSSCMGV	2.7793	55.66	51.86	52.12	53	2
P18	YSSGFCNIAV	8.0246	44.99	44.49	43.99	44.49	0.5
P19	LLVRNSFEV	0.3311	54.49	53.63	53.54	53.89	0.5
P20	ILWRQDIHLGV	0.5683	53.67	53.64	53.17	53.49	0.3
P21	MFCQLAKTYPV	8.2582	45.89	43.71	43.30	44	1
P22	LLVRNSFEVRV	2.4775	44.54	44.57	44.90	44.67	0.2
P23	IDILWRQDIHL	1.3789	46.06	46.07	45.94	46.02	0.1
P24	KLVVVGADGV	5.1555	46.04	45.81	45.63	45.83	0.2

P25	GMNWRPILTI	2.0614	42.29	42.23	43.29	42.60	0.6
P26	ILCATYVKV	0.8264	59.41	56.53	56.68	58	2
P27	LLGRNSFEVLV	2.9094	42.95	43.32	43.17	43.15	0.2
P28	LLDILDTAGL	2.519	41.15	41.34	41.16	41.22	0.1
P29	CLLDILDTAGL	1.5036	47.66	47.53	47.31	47.50	0.2
P30	FSGEYIPTV	0.1292	54.98	54.92	54.83	54.91	0.1
P31	RPLAWGNINL	1.6506	43.17	44.39	44.35	43.97	0.7
P32	ILNREIDFAI	1.5315	45.14	44.35	45.08	44.86	0.4
P33	GLKDLLNPI	0.4904	53.56	54.05	52.91	53.51	0.6
P34	YLDSGIHCGA	0.4688	51.57	52.00	52.04	51.87	0.3
P35	FCQLAKTYPV	3.1666	49.56	50.05	51.01	50.21	0.7
P36	ILWRQDIHL	0.0599	54.70	56.11	55.87	55.56	0.8
P37	GLVDEQQEV	0.0256	54.22	54.09	54.71	54.34	0.3
P38	NLLVRNSFEV	2.7092	46.73	48.28	47.86	47.62	0.8
P39	KLVVVGAVGV	2.8794	48.60	49.82	49.19	49.20	0.6
P40	KILCATYVKV	3.7401	46.86	48.63	48.17	47.89	0.9
P41	YLSTDVGFCTL	1.5797	49.73	50.14	51.12	50.33	0.7
P42	FMKQMNDAL	0.8267	43.05	43.87	45.95	44	1
P43	YLDSGIHFGA	0.282	54.34	54.38	54.49	54.40	0.1
P44	GLAPPQHLTRV	0.2137	54.84	56.42	54.23	55	1
P45	ALNNMFCQL	0.5605	51.43	52.48	50.14	51	1
P46	LLGRNSFEM	2.0018	46.81	49.04	48.27	48	1
P47	GLKDLLNPIGV	0.7564	48.25	48.64	50.17	49	1
P48	CQLAKTYPV	1.6839	55.87	55.34	56.51	55.91	0.6
P49	VLHECNSSYI	2.977	46.26	46.59	46.85	46.57	0.3
P50	KLVVVGAGCV	5.9357	48.07	46.72	48.03	47.61	0.8
T7A	SLLMWIAQV	0.0824	64.34	64.00	63.90	64.1	0.2
T7C	SLLMWICQV	0.2439	49.42	49.52	49.51	49.5	0.1
T7D	SLLMWIDQV	0.0765	60.20	60.21	60.02	60.1	0.1
T7E	SLLMWIEQV	0.032	58.76	58.93	60.18	59.3	0.8
T7F	SLLMWIFQV	0.0813	47.22	47.38	47.26	47.3	0.1
T7G	SLLMWIGQV	0.4506	57.87	57.29	57.39	57.5	0.3
T7H	SLLMWIHQV	0.01	63.54	63.40	63.24	63.4	0.2
T7I	SLLMWIIQV	0.0621	48.13	47.98	47.85	48.0	0.1
T7K	SLLMWIKQV	0.0912	57.43	57.42	57.25	57.4	0.1
T7L	SLLMWILQV	0.0766	45.09	46.38	47.21	46	1
T7M	SLLMWIMQV	0.0637	53.31	52.65	52.67	52.9	0.4
T7N	SLLMWINQV	0.0437	60.80	62.81	62.88	62	1

T7P	SLLMWIPQV	0.0191	65.32	65.42	65.64	65.5	0.2
T7Q	SLLMWIQQV	0.0197	59.99	59.84	60.08	59.97	0.12
T7R	SLLMWIRQV	0.1075	54.11	55.65	56.03	55	1
T7S	SLLMWISQV	0.0627	61.24	61.04	60.81	61.03	0.22
T7V	SLLMWIVQV	0.0473	45.68	46.81	46.73	46.4	0.6
T7W	SLLMWIWQV	0.0652	49.45	49.62	49.62	49.6	0.1
T7Y	SLLMWIYQV	0.0507	53.15	59.66	59.22	57	4
Q8A	SLLMWITAV	0.0985	59.71	59.72	59.69	59.71	0.02
Q8C	SLLMWITCV	0.328	50.36	50.45	50.07	50.3	0.2
Q8D	SLLMWITDV	0.2917	53.72	54.03	54.68	54.1	0.5
Q8E	SLLMWITEV	0.0271	57.20	55.73	55.17	56	1
Q8F	SLLMWITFV	0.1586	48.29	50.96	51.86	50	2
Q8G	SLLMWITGV	0.0923	70.61	69.49	69.62	69.9	0.6
Q8H	SLLMWITHV	0.0403	60.86	62.69	63.50	62	1
Q8I	SLLMWITIV	0.4052	65.58	57.96	56.95	60	5
Q8K	SLLMWITKV	0.0362	58.76	58.30	58.13	58.4	0.3
Q8L	SLLMWITLV	0.1587	49.31	48.97	48.61	49.0	0.4
Q8M	SLLMWITMV	0.1549	56.36	55.66	55.48	55.8	0.5
Q8N	SLLMWITNV	0.0586	62.31	62.23	61.63	62.1	0.4
Q8P	SLLMWITPV	0.2023	70.26	68.60	69.06	69.3	0.9
Q8R	SLLMWITRV	0.0546	58.99	58.77	58.49	58.8	0.3
Q8S	SLLMWITSV	0.0457	66.53	67.71	68.06	67.4	0.8
Q8T	SLLMWITTV	0.0484	63.55	63.90	63.82	63.8	0.2
Q8V	SLLMWITVV	0.2607	57.18	56.43	56.17	56.6	0.5
Q8W	SLLMWITWV	0.4367	53.03	53.26	53.18	53.2	0.1
Q8Y	SLLMWITYV	0.0739	60.66	58.97	60.01	59.9	0.9
S1A	ALLMWITQV	0.0284	53.99	52.51	51.38	53	1
S1C	CLLMWITQV	0.7334	49.30	49.35	49.27	49.31	0.04
S1D	DLLMWITQV	0.7663	45.40	45.64	45.13	45.4	0.3
S1E	ELLMWITQV	0.291	50.35	50.34	50.24	50.3	0.1
S1F	FLLMWITQV	0.0473	47.64	46.53	45.66	47	1
S1G	GLLMWITQV	0.0473	59.18	56.81	56.31	57	2
S1H	HLLMWITQV	0.0425	57.84	58.65	58.11	58.2	0.4
S1I	ILLMWITQV	0.0561	46.68	45.81	44.94	45.8	0.9
S1K	KLLMWITQV	0.0183	49.97	49.62	50.22	49.9	0.3
S1L	LLLMWITQV	0.1189	46.91	46.16	45.49	46.2	0.7
S1M	MLLMWITQV	0.0935	49.23	47.55	46.75	48	1
S1N	NLLMWITQV	0.0636	55.93	52.58	51.88	53	2

S1P	PLLMWITQV	0.9375	49.56	49.53	49.27	49.5	0.2
S1Q	QLLMWITQV	0.0703	53.16	53.05	52.85	53.0	0.2
S1R	RLLMWITQV	0.0299	49.01	49.66	49.58	49.4	0.4
S1T	TLLMWITQV	0.0466	51.89	52.78	52.38	52.4	0.4
S1V*	VLLMWITQV	0.0599					
S1W	WLLMWITQV	0.3532	46.60	46.32	46.12	46.3	0.2
S1Y	YLLMWITQV	0.028	53.95	52.71	50.68	52	2
M4A	SLLAWITQV	0.0054	50.68	51.46	51.46	51.2	0.5
M4C	SLLCWITQV	0.0629	60.40	60.83	60.55	60.6	0.2
M4D	SLLDWITQV	0.0007	48.43	48.96	48.47	48.6	0.3
M4E	SLL EWITQV	0.0008	49.37	49.01	48.79	49.1	0.3
M4F	SLLFWITQV	0.025	53.85	54.75	54.82	54.5	0.5
M4G	SLLGWITQV	0.0022	51.34	51.02	50.53	51.0	0.4
M4H	SLLHWITQV	0.0046	50.29	49.97	49.48	49.9	0.4
M4I	SLLIWITQV	0.0267	47.93	47.23	47.07	47.4	0.5
M4K	SLLKWITQV	0.0034	61.25	64.05	64.86	63	2
M4L	SLLLWITQV	0.0216	50.43	51.35	51.13	51.0	0.5
M4N	SLLNWITQV	0.0034	50.09	50.04	50.00	50.0	0.0
M4P	SLLPWITQV	0.001	50.87	51.21	52.36	51.5	0.8
M4Q	SLLQWITQV	0.0036	55.22	55.41	55.50	55.4	0.1
M4R	SLLRWITQV	0.0081	63.70	63.01	63.34	63.4	0.3
M4S	SLLSWITQV	0.0031	47.68	47.88	47.70	47.8	0.1
M4T	SLLTWITQV	0.0046	58.09	58.39	57.87	58.1	0.3
M4V	SLLVWITQV	0.0208	47.19	46.93	46.72	46.9	0.2
M4W	SLLWWITQV	0.0253	57.98	58.58	57.65	58.1	0.5
M4Y	SLLYWITQV	0.0187	60.66	59.91	57.62	59	2
W5A	SLLMAITQV	0.0438	49.97	50.54	50.51	50.3	0.3
W5C	SLLMCITQV	0.2115	60.99	59.22	58.44	60	1
W5D	SLLMDITQV	0.0209	57.28	56.76	56.46	56.8	0.4
W5E	SLLMEITQV	0.0156	60.16	60.36	60.21	60.2	0.1
W5F	SLLMFITQV	0.0319	55.93	54.43	53.63	55	1
W5G	SLLMGITQV	0.018	58.96	58.17	58.16	58.4	0.5
W5H	SLLMHITQV	0.0088	64.49	64.00	62.13	64	1
W5I	SLLMIITQV	0.0377	51.06	50.90	50.53	50.8	0.3
W5K	SLLMKITQV	0.0122	59.83	59.83	59.80	59.8	0.0
W5L	SLLMLITQV	0.0524	54.29	55.37	54.14	54.6	0.7
W5M	SLLMMITQV	0.0724	53.33	53.33	53.44	53.4	0.1
W5N	SLLMNITQV	0.0293	59.50	58.57	56.49	58	2

W5P	SLLMPITQV	0.025	55.97	57.71	57.59	57	1
W5Q	SLLMQITQV	0.0172	51.55	53.30	53.66	53	1
W5R	SLLMRITQV	0.0272	58.89	58.47	59.08	58.8	0.3
W5S	SLLMSITQV	0.0307	61.21	60.02	59.45	60.2	0.9
W5T	SLLMTITQV	0.0429	60.29	61.17	60.95	60.8	0.5
W5V	SLLMVITQV	0.0362	52.04	51.73	52.71	52.2	0.5
W5Y	SLLMYITQV	0.0201	57.21	57.93	57.70	57.6	0.4

Supplementary Table 4. Crystallography Data Collection and Refinement Statistics

Molecule	HLA-A*02:01/NY-ESO-1/MAM-Tracer
Data Collection	
PDB entry	8SUH
Beamline	NSLS II 17-ID-2 (FMX)
Space Group	P 2 ₁ 2 ₁ 2 ₁
Unit Cell	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	49.42 95.61 259.70
α , β , γ (°)	90.00 90.00 90.00
Wavelength (Å)	0.92
Resolution (Å) ¹	2.3 (2.37-2.3)
Unique reflections	55296 (5513)
Mean I/ σ (I) ²	5.7 (1.1)
CC 1/2	0.985 (0.444)
Completeness (%) ³	98.5 (99.9)
R _{merge}	0.282 (1.717)
R _{meas}	0.308 (1.877)
R _{pim}	0.122 (0.747)
Refinement	
Resolution range (Å)	89.73 - 2.3
R _{work} /R _{free} (%) ⁴	20.3 / 26.5
Num. of Copies Asym. Unit	2
Non-Hydrogen atom	
Macromolecules	8,343
Ligands	15
Solvent	531
Reflections used	55,274
R.m.s.d. ⁵	
Bond lengths (Å)	0.008
Bond angles (°)	1.06
MolProbity Score ⁶	1.99
Classscore ⁶	5.33
Ramachandran (%)	
Favored	96.96
Allowed	2.43
Outliers	0.61
Average B-factor	34.47
Macromolecules	34.38
Ligands	53.36
Solvent	35.28

¹Statistics for highest resolution bin of reflections in parentheses.

²Intensity signal-to-noise ratio.

³Completeness of the unique diffraction data.

⁴R_{free} is calculated against a 5% random sampling of the reflections that were removed before structure refinement.

⁵Root mean square deviation of bond lengths and bond angles.

⁶Chen et al. (2010) MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallographica D66:12-21.

Supplementary Table 5. Binding kinetics summary of monomeric *TRACeRs*.

<i>TRACeR</i>	K_D (M)	K_D error	kon	kon error	kdis	kdis error
HLA-A02/ NY-ESO-1 (Original redesign from dimer construct)	1.14e-7	1.34e-8	1.42e3	1.66e2	1.61e-4	1.61e-6
HLA-A02/ NY-ESO-1 (re-selected from the monomeric <i>TRACeR</i> library)	6.55e-7	1.60e8	2.52e3	2.90e1	1.65e-3	1.28e-5
HLA-A02/ EBV	3.13e-7	2.22e-8	7.69e4	5.26e3	2.41e-2	4.34e-4
HLA-A11/ KRAS G12V	2.16e-6	3.43e-8	3.31e2	3.47e0	7.15e-4	8.49e-6
HLA-A24/ Nef	4.18e-6	2.08e-7	6.65e4	1.25e3	2.78e-1	2.24e-3
HLA-B08/ SARS-CoV-2	1.43e-6	1.15e-7	1.69e5	1.30e4	2.41e-1	6.03e-3
HLA-C07/ PHOX2B	3.13e-6	7.78e-8	1.31e3	2.79e1	4.10e-3	5.17e-5

Supplementary Table 6. Sequence and convergence pattern for each target in the master library

Top 50 count sequences from the final round of sorted library are listed.

Library template: (? Denotes library position) SVEEIKKEYEERLKRFEDEFVERILKETGNKEIANMARMLLWRVERSIRYDKDNVELIKFNIRVIDWIMAEAEENDLCYFIGTHDKCENPKEQWVAN? QNL?NVV?TNKELEDYDESNKEETKEVLKKFKEKVNQF?EHAFDIINKYGDKEI?NMM?MLL?RV?RSFRIDANNVELIKFNIRVLDWIMAEADND LSYFISQ					
NYESO1-A02	EBV-A02	Kras_G12V-A11	Nef-A24	PHOX2B-C07	SAVS-CoV-2-B08
YNYQMWF	EWFTYVH	HDSRTMFQ	WTNNWGV	EYFWDDHD	KDHGIIVA
YNYDDWG	DLFWIGAD	TNGRTMFQ	WTNNFGKT	QNDWDDHD	AHEWWTHN
YNYDDWS	DLFTYVH	YDDRTMFQ	WYNTYGKM	QNDYWYED	DRSWWNVM
MNYQMWF	EWFWIGAD	KQVRTMFQ	WTNTYGKM	EYFYWYED	VWDWWDFFM
YNYQPYWT	TIWWIGRD	GHARTMFQ	WTNNWGT	EYFYDDHD	AHEGIIVA
MNYQPYWT	TIWETVH	DGTRTMFQ	WYNNWGV	EYFWWYED	KDHWVTHN
ANYQMWF	EWFWTYVH	DVARTMFQ	WYNNFGKT	QNDYDDHD	EHAWWAHT
PDEYDDWG	EWFTYFH	PHERTMFQ	WTNTYHEY	EYFYVYDE	NVHWWVHQ
YNYQDDWG	EWFWIGRD	EYTRTMFQ	WTNNWGKT	RTFWDDHD	NGVWWGMQ
PDEQMWF	EWFTYVN	GNARTMFQ	LIITYHEY	EAQWDDHD	GPKWWGTN
YNYMQWF	EWFTYVH	AEARTMFQ	WTNNYGKM	EYFYNYND	DRSWVTHN
YNYQDDWS	PWNETYVH	SPTRTMFQ	WTNNFGKM	QYFWDDHD	DRSGIIVA
GVAYDDWS	EYFTYVH	GIQRTMFQ	WTNNFGVT	EYFYWYHD	AHEWWNV

TMHYDDWG	PWNWIGAY	QDVRTMFQ	WTNTYGKT	RTFYVYDE	VWDWWTHN
YNYQMOWS	EYFWIGKD	ASFRTMFQ	WYNTYGKT	EYDWDDHD	KDHWVNVM
YNYQIQWF	EWFEIGAD	HQSRTMFQ	WTNTWGV	EAQYNYND	KDHGWTHN
GVAQMOWF	EWFETDVH	KPARTMFQ	LIINWGV	YDSWDDHD	NVHGIIA
FDDYDDWG	EQIETYVH	DSPTMFQ	LIINFGKT	EYFWDDHD	EHAWWTHN
YNFQMOWF	EQIWIAHD	NHIRTMFQ	WTNNYHEY	QNFWDDHD	AHEWIIA
MNYDVYFF	VFSETYVH	AIARTMFQ	WTNNWGKM	EYFWVYDE	VWDGIIVA
YNYQMOWG	TENYIAND	GYVRTMFQ	WTNTFGKT	QNDYWYHD	NVHWWTHN
TMHQMOWF	TENETYVH	GWLVTLQG	WYNTYHEY	EYFFDDHD	AHEWWAHT
YHYQMOWF	DWFETYVH	FSNRTMFQ	WYNTFGKT	EYFWNYND	AHEWWDFM
YNYDDWF	EWFWIGAY	TPFRTMFQ	WYNTWGV	EYFWDDQD	AHEWWVHQ
YNYQMQLF	EWFET_VH	RTKRTMFQ	WTNTWGTE	VASWDDHD	EHAGIIVA
ESRYDDWS	DLFWTYVH	GNGRTMFQ	LIITYGKM	EYFYNIDE	KDHWVWHQ
YNYQFYDA	TWFETYVH	YDSRTMFQ	WHNTWGTE	YDSYVYDE	KDHWWDFFM
FDDQMOWF	EWFWIGKD	KVKRTMFQ	WYNNWGTY	EYFFNYDD	KDHWWAHT
YHYQFYDA	HWGETYVH	HDDRTMFQ	WTNNWGTE	ENDWDDHD	GPKWVTHN
YNYQMOCF	VFSYIGYQ	TNARTMFQ	WYNTYGV	EYFYSYDE	NGVWVTHN
YYDQMOWF	EWFETYDH	HDARTMFQ	WTNTYGV	EYFWDYED	NGVGIIA
YNFQPQWT	YTLWIGHD	TNSRTMFQ	WHNNWGV	EYFYWDHD	GPKGIIA
YNYQPQWT	KHGETYVH	HDGRTMFQ	WTNNYGKT	EYFWDYDD	AHEWWGTN
YNYHADWI	EWFETYVA	HDTRTMFQ	WTNNFGTY	EYFYNIDE	AHEWWGMQ
ANYHADWI	EWFWIAHD	RTKRTMFQ	WHNNFGKT	EYFWNYDD	KDHWVGTN
INYQMOWF	EWFYTYVH	GQVRTMFQ	WYNNYGKM	EYFWWYHD	KDHWIIA
GQTYDDWS	YTLETYVH	TDSRTMFQ	WYNNFGVT	QNDWWYED	KDHWVGMQ
GQTQMOWF	AGYSTYLY	HNGRTMFQ	WTNAYHRY	EYFWDDDE	GNDWWSIT
YNYDVYFF	EWFEIGRD	KQARTMFQ	WYNNWGKT	VVEWDDHD	AHEWWTHT
GMDYDDWG	MTEWIARD	YDARTMFQ	WYNNFGKM	EYFWDDND	DRSWIIVA
ESRQMOWF	ESFWIGGD	YDGRTMFQ	WTNNFGEY	EYFWDDDD	PDMWVHHT
YNYQMOWT	KDGDVYVY	EIIRTMFQ	LIITFGKT	EYFWDDHE	KDHGWNVM
WISQMOWF	MPDYIGWD	TDIRTMFQ	WHNTYGKM	QNDYVYDE	EHAWWAHN
YNYQMHWF	DAPYIGDT	DDDRTMFQ	IAYAYHRY	VASYSYDE	DRSWVNVA
YYDYFFGG	NFFWLGQD	TNTRTMFQ	LIINWGV	QNDYWDHD	DRSWWDFM
HDIYDDWS	EYDVIGRG	KQGRTMFQ	LIITWGV	LGEWDDHD	AHHGIIVA
HDIQMOWF	VANWIGRD	FAIRTMFQ	WTNNYGV	RTFYDDHD	VWDWVNVM
GMDQMOWF	SIYWTGQD	TNGRTMFQ	WTNTWGV	EYDYWYED	RKDWVATQ
WISVYHAI	QVTYIGYG	DDSRTMFQ	WYNTWGTE	EYFWEDHD	DRSWVWHQ
YNYQMOWF	SYFWLAWD	HDSRTMFQ	WTNNFGKT	EYFWDDHD	VWDWIIVA

Appendix

Supplementary Appendix 1. PDB ID info for Fig. 1A

Representative TCR

1lp9 1mi5 1oga 1qrn 1qse 1qsf 1w72 2bnq 2bnr 2esv 2f53 2f54 2gj6 2j8u 2jcc 2p5e 2p5w 2pye 2uwe 2vlj
2vlk 2vlr 2ypl 3d3v 3d39 3dxa 3ffc 3gjf 3gsn 3h9s 3hae 3hgl 3kpr 3kps 3kxf 3mv7 3mv8 3mv9 3o4l
3pqy 3pwp 3qdg 3qdj 3qdm 3qeq 3qfj 3sjv 3uts 3utt 3vxn 3vyr 3vxs 3vxu 3w0w 4eup 4ftv 4g8g 4g9f
4jfd 4jfe 4jff 4l3e 4mji 4mnq 4mnb 4mxq 4n0c 4n5e 4qok 4qrp 4qrr 4wuu 5brz 5bs0 5c0a 5c0b 5c0c
5c07 5c08 5c09 5d2l 5d2n 5e6i 5e9d 5eu6 5euo 5hhm 5hho 5hyj 5isz 5ivx 5jhd 5jzi 5men 5nht 5nme
5nmf 5nmg 5nqk 5tez 5w1v 5w1w 5wkf 5wkh 5wlg 5xot 5xov 5yxn 5yxu 6am5 6amu 6avf 6avg 6bj2
6bj3 6bj8 6d78 6dkp 6eqa 6eqb 6mtm 6p64 6q3s 6r2l 6rp9 6rpa 6rpb 6rsy 6tmo 6tro 6uln 6ulr 6uon 6vm7
6vm8 6vm9 6vma 6vmc 6vmx 6vqo 6vrm 6vrn 6zkw 6zky 6zky 6zky 7dzm 7dzn 7edo 7jwi 7l1d 7n1e
7n1f 7n4k 7n5c 7n5p 7n6e 7na5 7ndq 7ndt 7ndu 7nme 7nmf 7nmg 7ow5 7ow6 7pb2 7pbc 7pdw 7q9a
7q9b 7q99 7qpj 7r80 7re7 7rk7 7rm4 7rrg 7rtr 7su9 8cx4 8gom 8gon 8gvb 8gvg 8gviedge cases (orange
and deep blue)
6vrn
6tro antibodies and non-canonical TCR in magenta
7re7
4wuu
1w72
7jwi (reverse polarity TCR)

Supplementary Appendix 2. PatchDock seeded RifDock flags setup for global docking step

Adapted from: Cao, L.X. et al. Design of protein-binding proteins from the target structure alone.
Nature **605**, 551-+ (2022).

```
#####RIFGEN OUTPUT#####  
-rif_dock:target_pdb      rifgen_NYESO1_lessres/rif_NYESO1_Tie2_sca0.8.rif.gz_target.pdb.gz  
-rif_dock:target_res      target_res.list  
-rif_dock:target_rf_resl   0.25  
-rif_dock:target_rf_cache  
rifgen_NYESO1_lessres/_RF_NYESO1_relaxed.pdb_CEN_trhash10651988_resl0.25_osamp2_replonly  
bdry  
-rif_dock:target_bounding_xmaps  
rifgen_NYESO1_lessres/rif_NYESO1_Tie2_sca0.8.rif.gz_BOUNDING_RIF_16.xmap.gz  
-rif_dock:target_bounding_xmaps  
rifgen_NYESO1_lessres/rif_NYESO1_Tie2_sca0.8.rif.gz_BOUNDING_RIF_08.xmap.gz  
-rif_dock:target_bounding_xmaps  
rifgen_NYESO1_lessres/rif_NYESO1_Tie2_sca0.8.rif.gz_BOUNDING_RIF_04.xmap.gz  
-rif_dock:target_bounding_xmaps  
rifgen_NYESO1_lessres/rif_NYESO1_Tie2_sca0.8.rif.gz_BOUNDING_RIF_02.xmap.gz  
-rif_dock:target_bounding_xmaps  
rifgen_NYESO1_lessres/rif_NYESO1_Tie2_sca0.8.rif.gz_BOUNDING_RIF_01.xmap.gz  
-rif_dock:target_rif      rifgen_NYESO1_lessres/rif_NYESO1_Tie2_sca0.8.rif.gz  
-rif_dock:extra_rotamers  0
```

```

-rif_dock:extra_rif_rotamers 1
-rif_dock:rot_spec_fname rifgen_NYESO1_lessres/rotamer_index_spec.txt
#####

-rif_dock:outdir rifdock_out_nyeso1
-rif_dock:dokfile all.dok
-rif_dock:n_pdb_out 30
-rif_dock:redundancy_filter_mag 1.0
-rif_dock:align_output_to_scaffold false
-output_full_scaffold
-beam_size_M 5
-hsearch_scale_factor 1.2
-rif_dock:global_score_cut -8.0
-rif_dock:seeding_pos "docking.res.txt"
-rif_dock:seeding_by_patchdock true
-rif_dock:patchdock_min_sasa 1000
-rif_dock:patchdock_top_ranks 2000
-rif_dock:xform_pos /home/users/dht14/patchdock/xform_pos_ang10.x
-rif_dock:cluster_score_cut -6.0
-rif_dock:keep_top_clusters_frac 1.0
-rosetta_score_each_seeding_at_least 1
-only_load_highest_resl
-n_pdb_out_global 30
-hbond_weight 2.0
-upweight_multi_hbond 0.0
-min_hb_quality_for_satisfaction -0.25
-scaff_bb_hbond_weight 2.0
-favorable_1body_multiplier 0.2
-favorable_1body_multiplier_cutoff 4
-favorable_2body_multiplier 5
-user_rotamer_bonus_constant 0
-user_rotamer_bonus_per_chi 0
-rif_dock:upweight_iface 2.0
-rif_dock::dont_use_scaffold_loops false
-rif_dock:scaffold_to_ala true
-rif_dock:scaffold_to_ala_selonly false
-rif_dock:replace_all_with_ala_1bre true
-hack_pack true
-rif_dock:hack_pack_frac 1.0
-rif_dock:rosetta_score_cut -10.0
-rif_dock:rosetta_score_fraction 1
-rif_dock:rosetta_min_fraction 1
-rif_dock:rosetta_min_at_least 30
-rif_dock:rosetta_min_at_most 300
-rif_dock:rosetta_score_at_most 3000
-rif_dock:replace_orig_scaffold_res false
-rif_dock:override_rosetta_pose true
-rif_dock:rosetta_min_scaffoldbb false
-rif_dock:rosetta_min_targetbb false
-rif_dock:rosetta_hard_min false
-rif_dock:rosetta_score_rifres_rifres_weight 0.6

```

```

-rif_dock:rosetta_score_rifres_scaffold_weight 0.4
-add_native_scaffold_rots_when_packing 0
-bonus_to_native_scaffold_res 0
-database /home/users/dht14/Rosetta/main/database
-beta
-score:weights beta_soft
-add_orbitals false
-rif_dock:pack_n_iters 2
-rif_dock:pack_iter_mult 2.0
-rif_dock:packing_use_rif_rotamers true
-rif_dock:extra_rotamers false
-rif_dock:always_available_rotamers_level 0
-rif_dock:rotrf_resl 0.25
-rif_dock:rotrf_spread 0.0
-rif_dock:rotrf_scale_atr 1.0
-rif_dock:rotrf_cache_dir /scratch/users/dht14/MHC-II_binder/rif_HA/rifdock-cache
-rif_dock::rf_resl 0.5
-rif_dock::rf_oversample 2
-rif_dock:use_scaffold_bounding_grids 0
-rif_dock:target_rf_oversample 2
-mute core.scoring.ScoreFunctionFactory
-mute core.io.pose_from_sfr.PoseFromSFRBuilder

```

Supplementary Appendix 3. Target_res.list for RifDock input

The model is based on PDB ID 1S9W, Residue number 1-180 was numbered the same as 1S9W, residue number >180 on MHC was truncated from the model because they are far from binding interface. Residue number 181-188 is the peptide (SLLMWITQV).

```

61
62
64
65
68
69
72
73
75
76
77
149
150
151
152
154
155
158
159
162
163
181
184

```

185
187
188

Supplementary Appendix 4. RosettaScripts for running interactive docking and interface design

```
<ROSETTASCRIPTS>
  <SCOREFXNS>
    <ScoreFunction name="sfxn" weights="beta_nov16" />
    <ScoreFunction name="sfxn_design" weights="beta_nov16" >
      <Reweight scoretype="approximate_buried_unsat_penalty" weight="5.0" />
      <Reweight scoretype="aa_composition" weight="1.0" />
    </ScoreFunction>
  </SCOREFXNS>
  <RESIDUE_SELECTORS>

    <Chain name="chainA" chains="A"/>
    <Chain name="chainB" chains="B"/>
    <Neighborhood name="interface_chA" selector="chainB" distance="8.0" />
    <Neighborhood name="interface_chB" selector="chainA" distance="8.0" />
    <And name="AB_interface" selectors="interface_chA,interface_chB" />
    <Not name="Not_interface" selector="AB_interface" />

    <ResidueName name="pro_and_gly_positions" residue_name3="PRO,GLY" />
    <ResidueName
      name="apolar"
      residue_name3="ALA,CYS,PHE,ILE,LEU,MET,THR,PRO,VAL,TRP,TYR" />
    <Not name="polar" selector="apolar" />
    <Index name="interaction_site" resnums="429,432,433,436,468,471,472" />
    <Index name="peptide" resnums="364,365,366,367,368,369,370,371,372,373,374,375,376" />
    <And name="interaction_site_interface" selectors="AB_interface,interaction_site"/>
    <Not name="not_site" selector="interaction_site_interface" />
    <And name="chainBnotSite" selectors="chainB,not_site"/>
    <Not name="not_peptide" selector="peptide" />
    <And name="MHC_not_peptide" selectors="chainB,not_peptide" />

    <!-- Layer Design -->
    <Index name="Nter" resnums="480" error_on_out_of_bounds_index="true" reverse="false" />
    <Index name="Cter" resnums="1" error_on_out_of_bounds_index="true" reverse="true" />
    <Layer name="surface" select_core="false" select_boundary="false" select_surface="true"
      use_sidechain_neighbors="true"/>
    <Layer name="boundary" select_core="false" select_boundary="true" select_surface="false"
      use_sidechain_neighbors="true"/>
    <Layer name="core" select_core="true" select_boundary="false" select_surface="false"
      use_sidechain_neighbors="true"/>
    <SecondaryStructure name="sheet" overlap="0" minH="3" minE="2"
      include_terminal_loops="false" use_dssp="true" ss="E"/>
    <SecondaryStructure name="entire_loop" overlap="0" minH="3" minE="2"
      include_terminal_loops="true" use_dssp="true" ss="L"/>
```



```

    <SecondaryStructure      name="entire_helix"      overlap="0"      minH="3"      minE="2"
include_terminal_loops="false" use_dssp="true" ss="H"/>
    <And name="helix_cap" selectors="entire_loop">
        <PrimarySequenceNeighborhood lower="1" upper="0" selector="entire_helix"/>
    </And>
    <And name="helix_start" selectors="entire_helix">
        <PrimarySequenceNeighborhood lower="0" upper="1" selector="helix_cap"/>
    </And>
    <And name="helix" selectors="entire_helix">
        <Not selector="helix_start"/>
    </And>
    <And name="loop" selectors="entire_loop">
        <Not selector="helix_cap"/>
    </And>
</RESIDUE_SELECTORS>
<TASKOPERATIONS>
    <ProteinInterfaceDesign name="pack_long" design_chain1="0" design_chain2="0" jump="1"
interface_distance_cutoff="15"/>

```

```

#####commonly used taskops#####
<InitializeFromCommandline name="init" />
<IncludeCurrent name="current" />
<LimitAromaChi2 name="limitchi2" chi2max="110" chi2min="70" include_trp="True" />
<ExtraRotamersGeneric name="ex1_ex2" ex1="1" ex2aro="1" />
<ExtraRotamersGeneric name="ex1" ex1="1" />
<ConsensusLoopDesign name="consensus_loop" />
#####Change a.a. preference of core layers#####
<DesignRestrictions name="layer_design">
    <Action selector_logic="Nter AND chainA" aas="ADEFGHIKLMNPQRSTVWY" />
    <Action selector_logic="Cter AND chainA" aas="ADEFGHIKLMNPQRSTVWY" />
    <Action selector_logic="surface AND helix_start AND chainA" aas="EHKPQRDNST"/>
    <Action selector_logic="surface AND helix AND chainA" aas="EHKQRDNST"/>
    <Action selector_logic="surface AND sheet AND chainA" aas="DEHKNQRST"/>
    <Action selector_logic="surface AND loop AND chainA" aas="DEGHKNPQRST"/>
    <Action selector_logic="boundary AND helix_start AND chainA" aas="ADEIKLMNPQRSTVWY"/>
    <Action selector_logic="boundary AND helix AND chainA" aas="ADEFIKLMNQRSTVWY"/>#add F for boundary, as pointed by Brian. In the original
LayerDesign setting, F is not allowed here.
    <Action selector_logic="boundary AND sheet AND chainA" aas="DEFIKLNQRSTVWY"/>
    <Action selector_logic="boundary AND loop AND chainA" aas="ADEFGIKLNQRSTVWY"/>
    <Action selector_logic="core AND helix_start AND chainA" aas="AFILMPVWY"/>
    <Action selector_logic="core AND helix AND chainA" aas="AFILMVWYDENQTS"/>
    <Action selector_logic="core AND sheet AND chainA" aas="FILMVWYDENQST"/># add M
    <Action selector_logic="core AND loop AND chainA" aas="AFGILMPVWY"/>
    <Action selector_logic="helix_cap AND chainA" aas="DNST"/>

```

```

        <Action selector_logic="interaction_site AND chainB"
aas="ADEFGHIKLMNPQRSTVWY" />
    </DesignRestrictions>

```

```

##### restrict to interface#####
    <SelectBySASA name="PR_monomer_core" mode="sc" state="monomer" probe_radius="2.2"
core_asa="15" surface_asa="15" core="0" boundary="1" surface="1" verbose="0" />
    <OperateOnResidueSubset name="restrict_to_interface" selector="Not_interface">
        <PreventRepackingRLT/>
    </OperateOnResidueSubset>
    <OperateOnResidueSubset name="restrict_target" selector="chainBnotSite">
        <PreventRepackingRLT/>
    </OperateOnResidueSubset>
    <OperateOnResidueSubset name="restrict_target2repacking" selector="chainBnotSite">
        <RestrictToRepackingRLT/>
    </OperateOnResidueSubset>

##### This is just to prevent repacking, not constrains #####
    <DisallowIfNonnative name="disallow_GLY" resnum="0" disallow_aas="G" />
    <DisallowIfNonnative name="disallow_PRO" resnum="0" disallow_aas="P" />
    <OperateOnResidueSubset name="restrict_PRO_GLY" selector="pro_and_gly_positions">
        <PreventRepackingRLT/>
    </OperateOnResidueSubset>
    <ProteinProteinInterfaceUpweighter name="up_ppi" interface_weight="2" skip_loop_in_chain="A"
/>

```

```

</TASKOPERATIONS>

```

```

<MOVERS>

```

```

    <SwitchChainOrder name="chain2onlypre" chain_order="2" />
    <ScoreMover name="scorepose" scorefxn="sfxn" verbose="false" />
    <ParsedProtocol name="chain2only">
        <Add mover="chain2onlypre" />
        <Add mover="scorepose" />
    </ParsedProtocol>
    <DeleteRegionMover name="only_peptide" residue_selector="MHC_not_peptide" rechain="false"/>
    <TaskAwareMinMover name="min" scorefxn="sfxn" bb="0" chi="1" task_operations="pack_long"
/>

```

```

</MOVERS>

```

```

<FILTERS>

```

```

    <Sasa name="interface_buried_sasa" confidence="0" />
    <ShapeComplementarity name="interface_sc" verbose="0" min_sc="0.55" write_int_area="1"
write_median_dist="1" jump="1" confidence="0"/>
    <Ddg name="ddg_to_peptide" threshold="-5" jump="1" repeats="3" repack="1" relax_mover="min"
confidence="1" scorefxn="sfxn" />
    <MoveBeforeFilter name="ddg_peptide" mover="only_peptide" filter="ddg_to_peptide"
confidence="1"/>
    <BuriedUnsatHbonds name="vbuns_heavy_all" use_reporter_behavior="true"
report_all_heavy_atom_unsats="true" scorefxn="sfxn" residue_selector="AB_interface"
ignore_surface_res="false" print_out_info_to_pdb="true" confidence="1" cutoff="6"

```

```

use_ddG_style="true" dalphaball_sasa="true" probe_radius="1.1" atomic_depth_selection="5.5"
burial_cutoff="1000" burial_cutoff_apo="0.2" />
  <BuriedUnsatHbonds name="vbuns_heavy_sc" use_reporter_behavior="true"
report_sc_heavy_atom_unsats="true" scorefxn="sfxn" residue_selector="AB_interface"
ignore_surface_res="false" print_out_info_to_pdb="true" confidence="0" use_ddG_style="true"
dalphaball_sasa="true" probe_radius="1.1" atomic_depth_selection="5.5" burial_cutoff="1000"
burial_cutoff_apo="0.2" />
  <BuriedUnsatHbonds name="vbuns_heavy_bb" use_reporter_behavior="true"
report_bb_heavy_atom_unsats="true" scorefxn="sfxn" residue_selector="AB_interface"
ignore_surface_res="false" print_out_info_to_pdb="true" confidence="0" use_ddG_style="true"
dalphaball_sasa="true" probe_radius="1.1" atomic_depth_selection="5.5" burial_cutoff="1000"
burial_cutoff_apo="0.2" />
  ### score function monomer terms

</FILTERS>
<MOVERS>
  <AddCompositionConstraintMover name="add_aa_composition" selector="chainA">
    <Comp entry="PENALTY_DEFINITION;TYPE ALA;ABSOLUTE 0;PENALTIES 0
3;DELTA_START 0;DELTA_END 1;BEFORE_FUNCTION CONSTANT;AFTER_FUNCTION
LINEAR;END_PENALTY_DEFINITION;" />
  </AddCompositionConstraintMover>
  <ClearCompositionConstraintsMover name="clear_aa_composition" />
  <Docking name="docking" score_high="soft_rep" fullatom="0" local_refine="0"/>
  <Docking name="docking_high" score_high="soft_rep" fullatom="1" local_refine="1"/>
  <FastDesign name="FastDesignMonomer1" scorefxn="sfxn" repeats="1"
task_operations="init,current,layer_design,restrict_target2repacking,consensus_loop" >
    <MoveMap name="MM" >
      <Chain number="1" chi="true" bb="false" />
      <Chain number="2" chi="true" bb="true" />
      <Jump number="1" setting="true" />
    </MoveMap>
  </FastDesign>
  <FastDesign name="FastDesignMonomer2" scorefxn="sfxn_design" repeats="2"
task_operations="init,current,layer_design,restrict_target2repacking,consensus_loop,ex1_ex2,ex1" >
    <MoveMap name="MM" >
      <Chain number="1" chi="true" bb="false" />
      <Chain number="2" chi="true" bb="true" />
      <Jump number="1" setting="true" />
    </MoveMap>
  </FastDesign>
</MOVERS>

<APPLY_TO_POSE>
</APPLY_TO_POSE>
<PROTOCOLS>
  <Add mover="FastDesignMonomer1" />
  <Add mover="docking" />
  <Add mover="add_aa_composition" />
  <Add mover="FastDesignMonomer2" />
  <Add mover="clear_aa_composition" />
  <Add mover="docking_high" />

```

```

    <Add filter_name="ddg_peptide" />
    <Add filter_name="interface_sc" />
        <Add filter_name="vbuns_heavy_all" />
        <Add filter_name="vbuns_heavy_sc" />
        <Add filter_name="vbuns_heavy_bb" />
</PROTOCOLS>
<OUTPUT />
</ROSETTASCRIPTS>

```

Supplementary Appendix 5. Script to filter Rosetta outputs on evaluation metrics and sort outputs based on binding energy

Evaluation filters: Shape complementary score > 0.6, buried unsatisfied hydrogen bond < 3, contact area > 420.

All outputs are sorted on binding energy ddg and top100 are selected for guiding library design.

```

import os
input_dir = './'
output_dir = input_dir + 'top1000/'
output_filename = 'ranked_list'
file_num = 1000
output = open(output_dir + output_filename, 'w')

arr=[]
arr2=[]
count = 0
for file in os.listdir(input_dir): # runs/170202_tim10_short_csts
    if file.endswith('.pdb') and 'design' in file:
        file_loc = input_dir + str(file)
        op_file = open(file_loc)
        count +=1
        new_entry = [file]
        print(count)
        for line in op_file:
            if 'ddg' in line:
                sp_line = line.split(' ')
                score = sp_line[1] # got score
                new_entry.append(float(score))
            elif 'interface_sc ' in line:
                line.strip('\n')
                sp_line2 = line.split(' ')
                isc = sp_line2[1]
                new_entry.append(float(isc))
            elif 'score_per_res' in line:
                line.strip('\n')
                sp_line3 = line.split(' ')
                spr = sp_line3[1]
                new_entry.append(float(spr))
            elif 'vbuns_heavy_all ' in line:

```

```

        line.strip('\n')
        sp_line4 = line.split(' ')
        vha = sp_line4[1]
        new_entry.append(int(vha))
    elif 'vbuns_heavy_bb' in line:
        line.strip('\n')
        sp_line5 = line.split(' ')
        vhb = sp_line5[1]
        new_entry.append(int(vhb))
    elif 'vbuns_heavy_sc' in line:
        line.strip('\n')
        sp_line6 = line.split(' ')
        vhs = sp_line6[1]
        new_entry.append(int(vhs))
    elif 'contact_area_binder' in line:
        line.strip('\n')
        sp_line7 = line.split(' ')
        cab = sp_line7[1]
        new_entry.append(float(cab))
    elif 'contact_area_target' in line:
        line.strip('\n')
        sp_line8 = line.split(' ')
        cat = sp_line8[1]
        new_entry.append(float(cat))
    if float(spr) < -3 and float(cab) > 420 and float(cat) > 420 and int(vha) < 3 and float(isc) > 0.6:
        print(new_entry)
        arr.append(new_entry)
    op_file.close()
    #break
print(arr)
arr = sorted(arr, key=lambda x: x[3])
print(arr)
#print(arr2)
for i in range(0, file_num):
    output.write(str(i+1) + ' ' + str(arr[i][0]) + ' ' + str(arr[i][1]) + ' ' + str(arr[i][2]) + ' ' + str(arr[i][3]) + ' ' +
    str(arr[i][4]) + ' ' + str(arr[i][5]) + ' ' + str(arr[i][6]) + ' ' + str(arr[i][7]) + ' ' + str(arr[i][8]) + '\n')
    command = 'cp ' + input_dir + arr[i][0] + ' ' + output_dir + str(i + 1) + '.pdb'
    print(command)
    os.system(command)

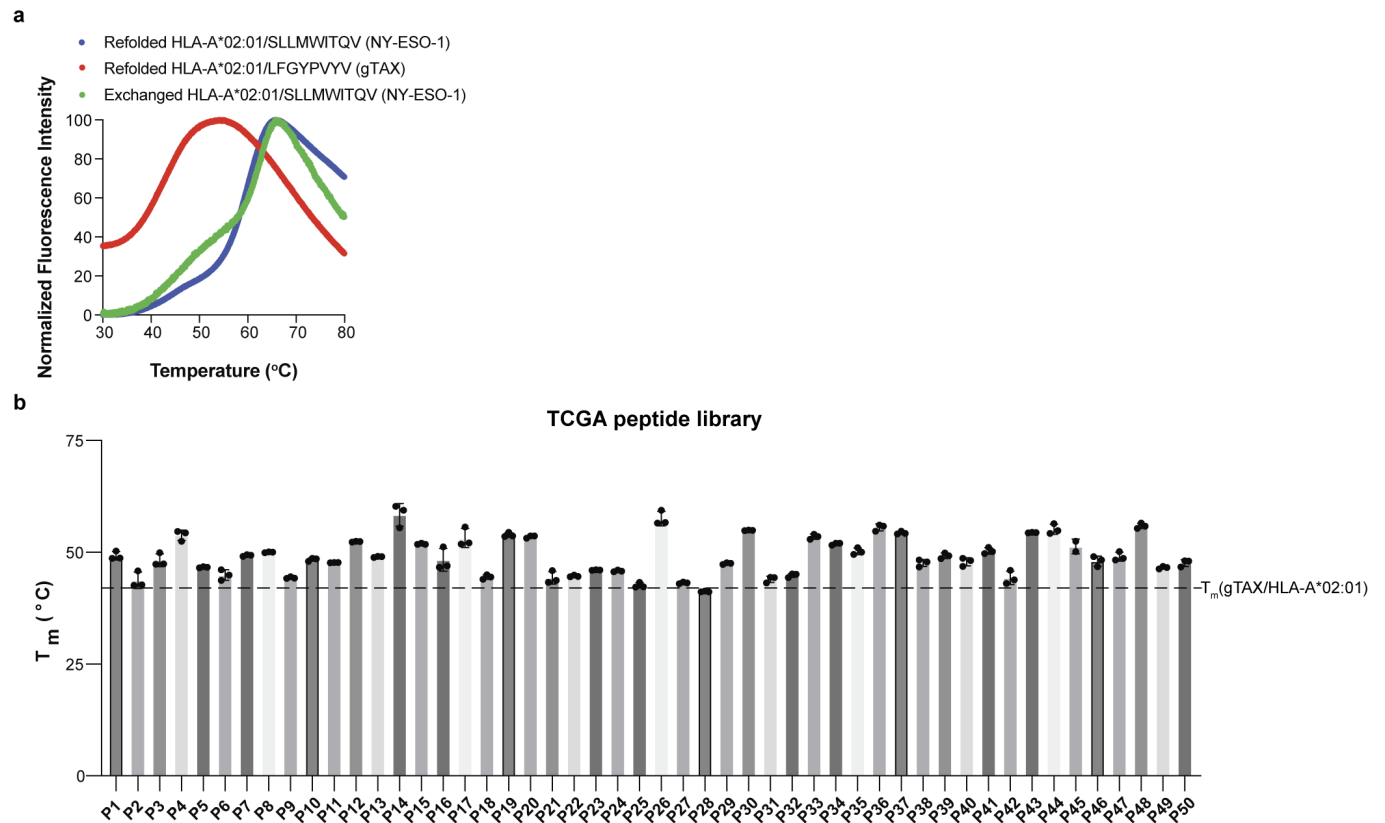
output.close()

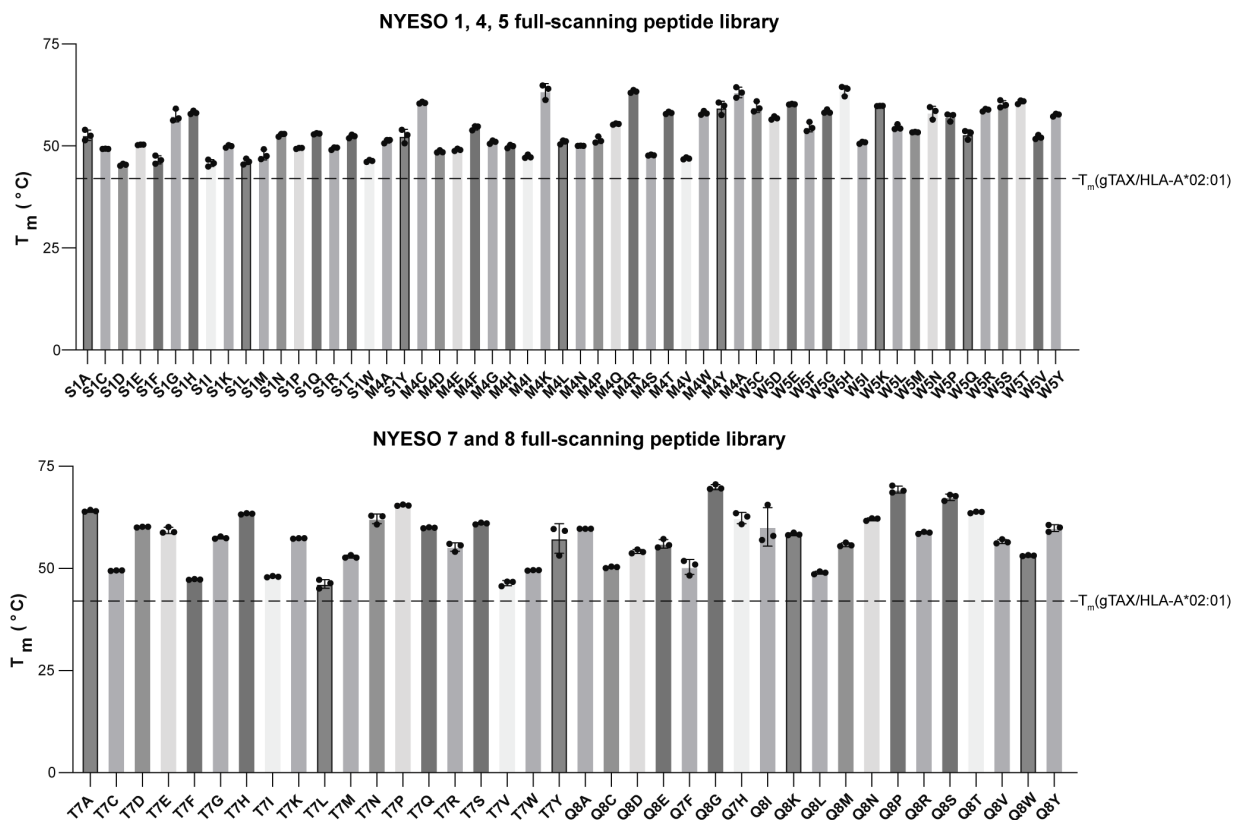
```

Supplementary Appendix 6. Peptide loading was confirmed for individual pHLA-A*02:01 tetramer

a. Thermal stability curves of refolded NY-ESO-1/HLA-A*02:01 (blue), refolded gTAX/HLA-A*02:01 (red), and exchanged NY-ESO-1/HLA-A*02:01 (refolded gTAX/HLA-A*02:01 in the presence of 10-fold molar excess NY-ESO-1 peptide and molecular chaperone TAPBPR) measured by differential scanning fluorimetry (DSF). **b. and c.** Melting temperature (T_m , °C)

obtained from thermal stability curves of HLA-A*02:01 bound to individual peptides from TCGA (b) and NY-ESO-1 1, 4, 5, 7, and 8 full-scanning (c) peptide libraries via TAPBPR-mediated peptide exchange. Upon replacing the place-holder peptide gTAX of refolded gTAX/HLA-A*02:01 with a moderate to high-affinity peptide, an increasing T_m was observed compared to the T_m of refolded gTAX/HLA-A*02:01. Data are mean $\pm$ SD for $n = 3$ technical replicates.





References

1. Barker, D.J., Maccari, G., Georgiou, X., Cooper, M.A., Flicek, P., Robinson, J., and Marsh, S.G.E. (2023). The IPD-IMGT/HLA Database. *Nucleic Acids Res* 51, D1053-D1060. [10.1093/nar/gkac1011](https://doi.org/10.1093/nar/gkac1011).
2. Dauparas, J., Anishchenko, I., Bennett, N., Bai, H., Ragotte, R.J., Milles, L.F., Wicky, B.I.M., Courbet, A., de Haas, R.J., Bethel, N., et al. (2022). Robust deep learning-based protein sequence design using ProteinMPNN. *Science* 378, 49-55. [10.1126/science.add2187](https://doi.org/10.1126/science.add2187).