

Supplementary Information

Single variable domains from the T cell receptor β chain function as mono- and bifunctional CARs and TCRs

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Supplementary Figure S1. Expression and target pMHC tetramer binding of V β domains

FACS plot of HEK293T cells transfected with V β -only domains recovered from the HuTARGTM system. The x-axis reflects the amount of target pMHC probes bound to cell. The y-axis reflects the amount of the TCR β chain on the cell surface.

Supplementary Figure S2. Constructs show target specific NFAT activation

NFAT-luciferase signal of transfected Jurkat cells after 6hrs of co-culture with NY-ESO-1 (blue), MAGE-A3 (red), or unrelated (green) peptides-loaded T2 cells. The error bars indicate SD (n=2).

Supplementary Figure S3. Idiotypes of MAGE-A3 V β -only binders show a range of background signal.

NFAT-luciferase signal of transfected Jurkat cells after 6hrs of co-culture with MAGE-A peptide-loaded T2 cells. The error bars indicate SD (n=2).

Supplementary Figure S4. Fluorescence microscopy of stained constructs

Transfected Jurkats cells were fixed and stained with cognate target pMHC tetramers conjugated with PE in the presence or absence of nonionic detergent for permeabilization. The cells were then analyzed with a 40x objective for cellular localization of CAR constructs.

Supplementary Figure S5. V β -only CARs with CD8 hinge

a, Schematics of the V β -only-CARs tested. **b**, NFAT-luciferase signal of transfected Jurkat cells after 6hrs of co-culture with NY-ESO-1 (left) and MAGE-A3 (right) peptide-loaded T2 cells. The error bars indicate SD (n=2).

Supplementary Figure S6. Alpha chain truncation

a, Surface expression of CD3 ϵ and target probe binding in SUP-T1 cells measured by FACS staining. **b**, NFAT-luciferase signal of transfected Jurkat cells after 6hrs of co-culture with NY-ESO-1 peptide-loaded T2 cells. The error bars indicate SD (n=2).

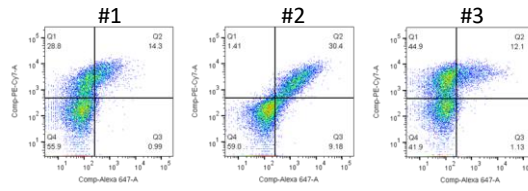
Supplementary Figure S7. scFv-TCR fusion follows scFv-CAR sensitivity

a, Schematics of the constructs tested. **b**, NFAT-luciferase signal of transfected Jurkat cells after 6hrs of co-culture with NY-ESO-1 peptide-loaded T2 cells. The error bars indicate SD (n=2).

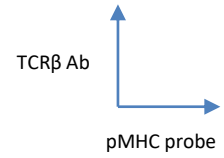
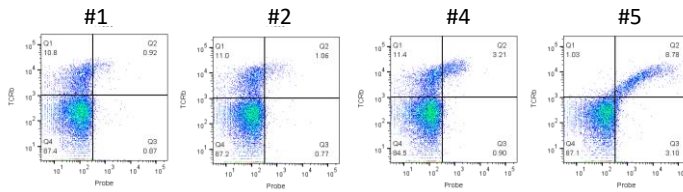
Supplementary Figure S8. Real-time killing assay with K562 target cells

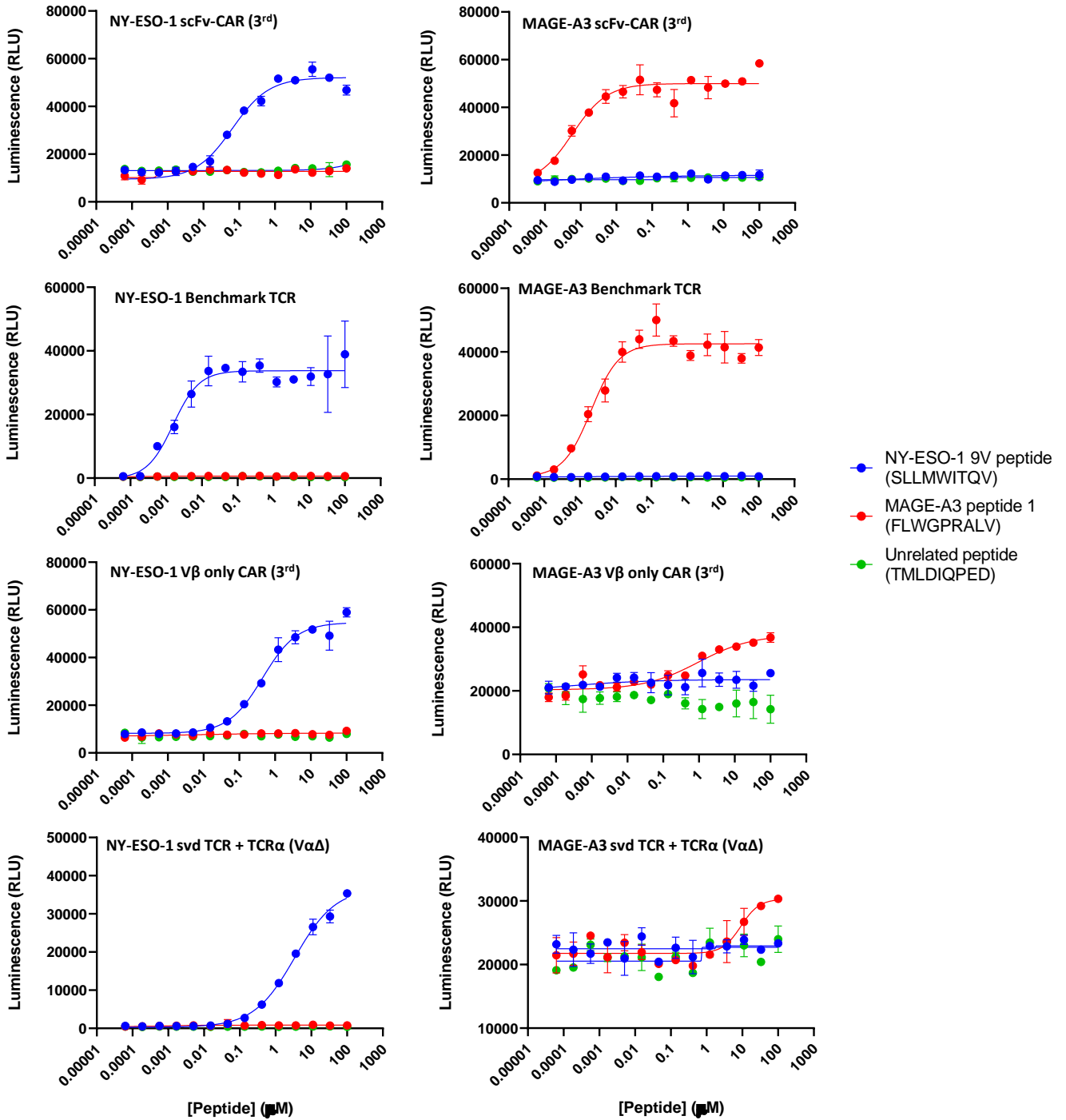
Related to Fig 6B. K562 cells overexpressing single chain NY-ESO-1- β ₂m-HLA-A2 trimer and GFP were co-cultured with T cells expressing NY-ESO-1 (left) or MAGE-A3 (right) targeted constructs at 1:1 ratios. Images were taken for 42 hours by IncuCyte. Total green fluorescent area at each time point divided by the t=0 value are plotted. The error bars indicate SD (n=2).

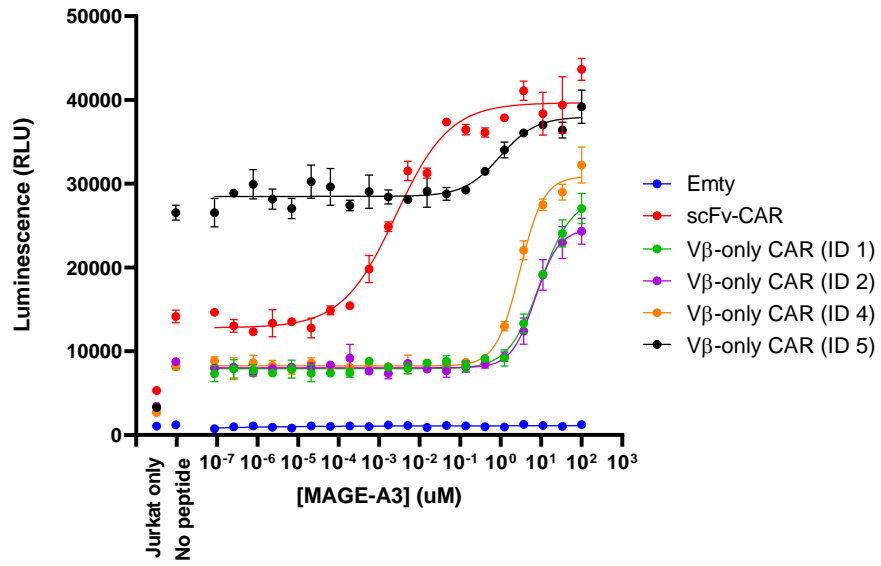
NY-ESO1 binders

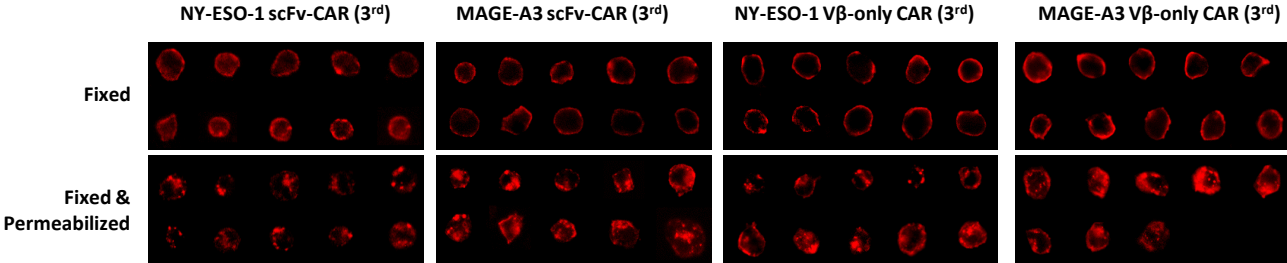


MAGE-A3 binders

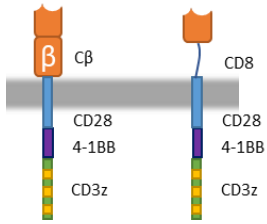




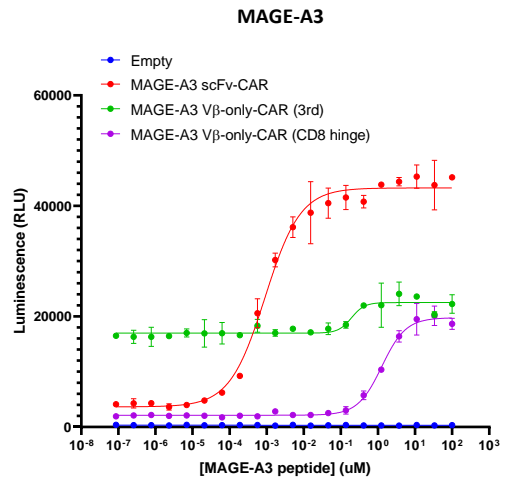
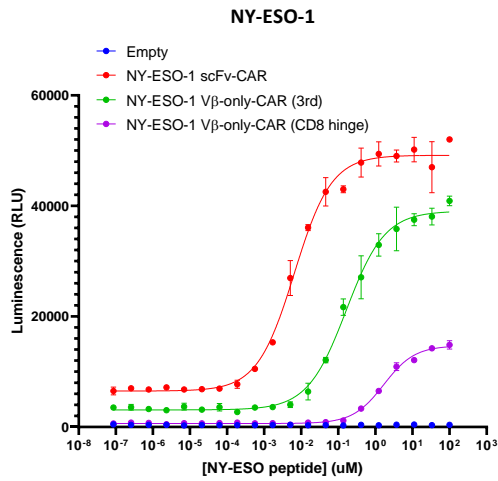




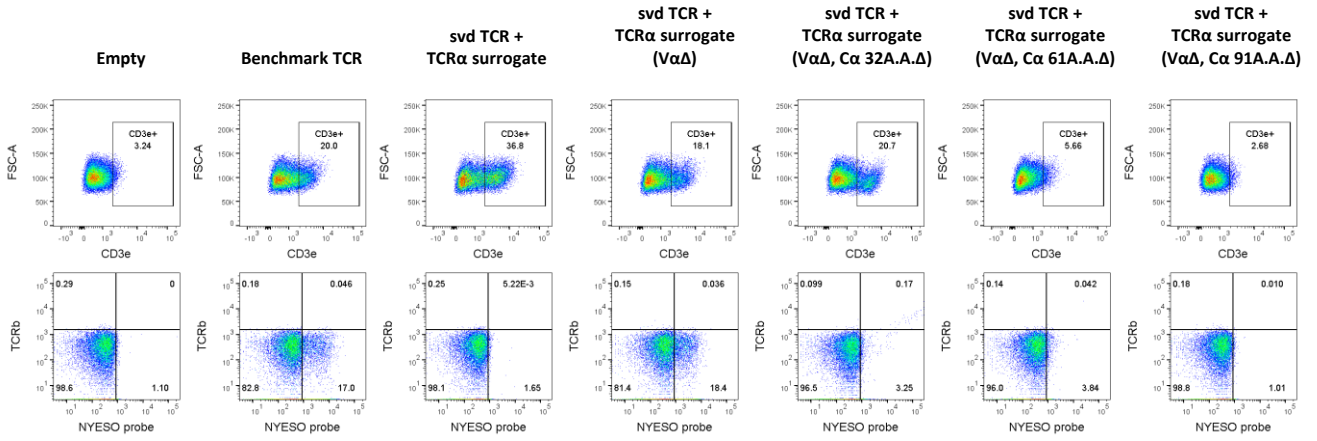
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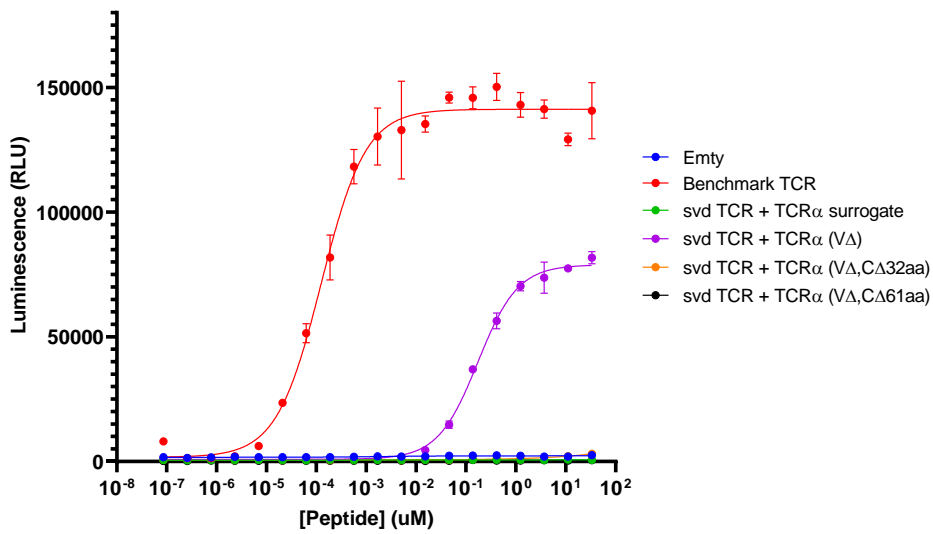
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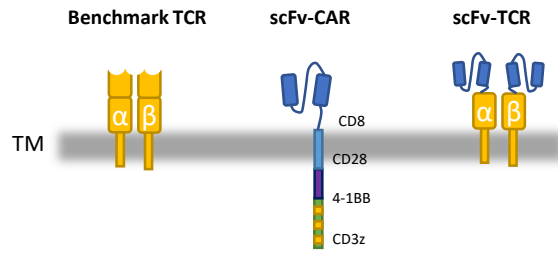
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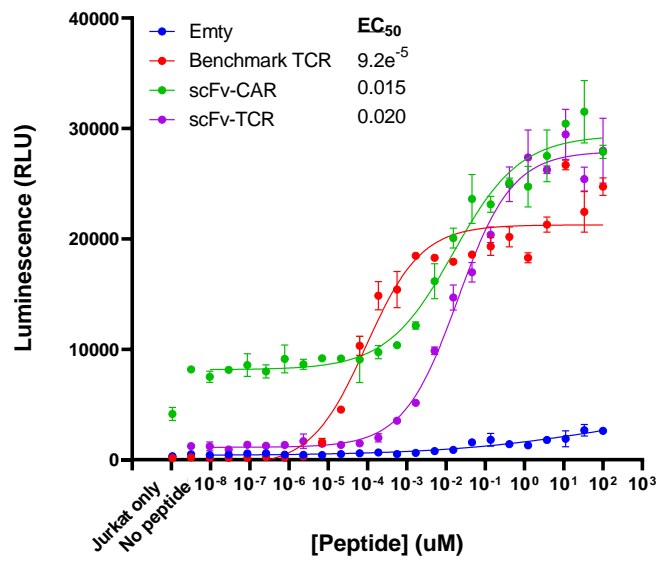
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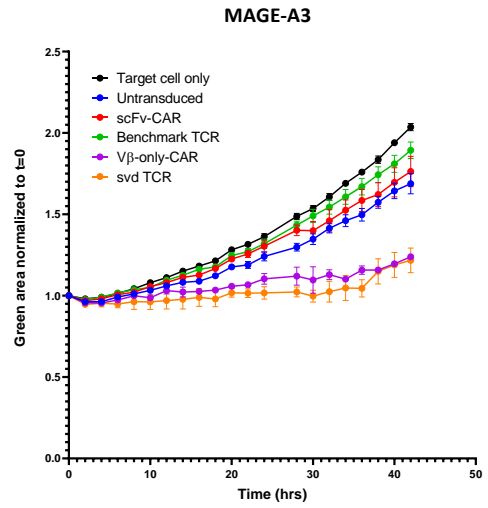
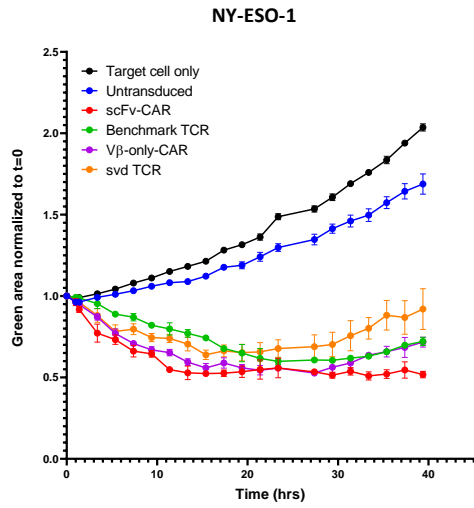


a



b





Supplementary Table 1. CDR3 sequence alignments of identified Vβ domains

Binder	ID#	TRBV Gene	TRBD Gene	TRBJ Gene	CDR3 Nucleotide Sequence	CDR3 Amino Acid Sequence
NY-ESO	1	TRBV5-8*01	N/A	TRBJ2-7*01	TGTGCCAGCAGCTTGGCCTCGGCTACGAGCAGTACTTC	CASSLGLGYEQYF
NY-ESO	2	TRBV5-8*01	TRBD2*02	TRBJ2-1*01	TGTGCCAGCAGCTTGGGGGTCCCGGGGACTAGCGGGACTTAGGGGGGATGAGCAGTTC	CASSLGGPRGLAGLRGDEQF
NY-ESO	3	TRBV5-8*01	TRBD2*01	TRBJ2-1*01	TGTGCCAGCAGCTTAAGGCGGGACAATGAGCAGTTC	CASSLRDNEQF
MAGE-A3	1	TRBV5-8*01	TRBD1*01	TRBJ2-3*01	TGTGCCAGCAGCTTGGAGTCCTGCTGGGGCGGATTTCCAGATACGAGTATTTT	CASSLEVLLGADFPDQYF
MAGE-A3	2	TRBV5-8*01	TRBD2*02	TRBJ2-1*01	TGTGCCAGCAGCTTCCGGCGGGACACGGGGCGGATCTGGACAATGAGCAGTTC	CASSFPAGHGADLDNEQF
MAGE-A3	4	TRBV5-8*01	TRBD1*01	TRBJ2-1*01	TGTGCCAGCAGCAGGAGATAACGGGGCGGATAGGGGAGCAGTTC	CASSEITGRIGEQF
MAGE-A3	5	TRBV5-8*01	TRBD1*01	TRBJ2-1*01	TGTGCCAGCAGCTTGGGGGGGACGAGCTGGGGCGGATGGGAATGAGCAGTTC	CASSLGGDELGADGNEQF

N/A = unassignable TRBD

BLUE: TRBV Sequence

BLACK: TRBD Sequence

PURPLE: TRBJ Sequence

RED: Product of terminal deoxynucleotidyl transferase (TDT)

Supplementary Table 2. EC₅₀ from NFAT Luciferase assay

Figures	Constructs	EC ₅₀ (mM)	
		NY-ESO-1 binders	MAGE-A3 binders
Fig. 3	Benchmark TCR	0.00006	0.002
Fig. 3	scFv-CAR	0.002	0.001
Fig. 3	svd TCR + TCRα surrogate	-	-
Fig. 3	Vβ-only CAR (CD3z)	0.210	1.2
Fig. 3	Vβ-only CAR (CD28-CD3z)	0.091	0.55
Fig. 3	Vβ-only CAR (CD28-41BB-CD3z)	0.100	0.99
Fig. 4C	scFv-CAR	0.008	0.001
Fig. 4C	svd TCR + TCRα surrogate	-	-
Fig. 4C	Vβ-only CAR (CD3z)	0.40	1.2
Fig. 4C	Vβ-only CAR (CD28-CD3z)	0.18	0.55
Fig. 4C	Vβ-only CAR (CD28-41BB-CD3z)	0.15	0.99
Fig. 4C	svd TCR + TCRα (VΔ)	0.83	2.7
Fig. 5B	Vβ-only CAR (CD28-41BB-CD3z)	0.16	0.19
Fig. 5B	N-M-Vβ-only CAR	3.0	0.28
Fig. 5B	M-N-Vβ-only CAR	0.37	1.8
Fig. 5D	svd TCR + TCRα (VΔ)	0.24	13
Fig. 5D	N-M-svd TCR + TCRα (VΔ)	0.21	4.7
Fig. 5D	M-N-svd TCR + TCRα (VΔ)	1.6	16