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Deciphering Membrane-Protein Interactions and High-Throughput Antigen Identification with Cell Doublets

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Supplementary Figures for

Deciphering membrane-protein interactions and high-throughput antigen identification with cell doublets

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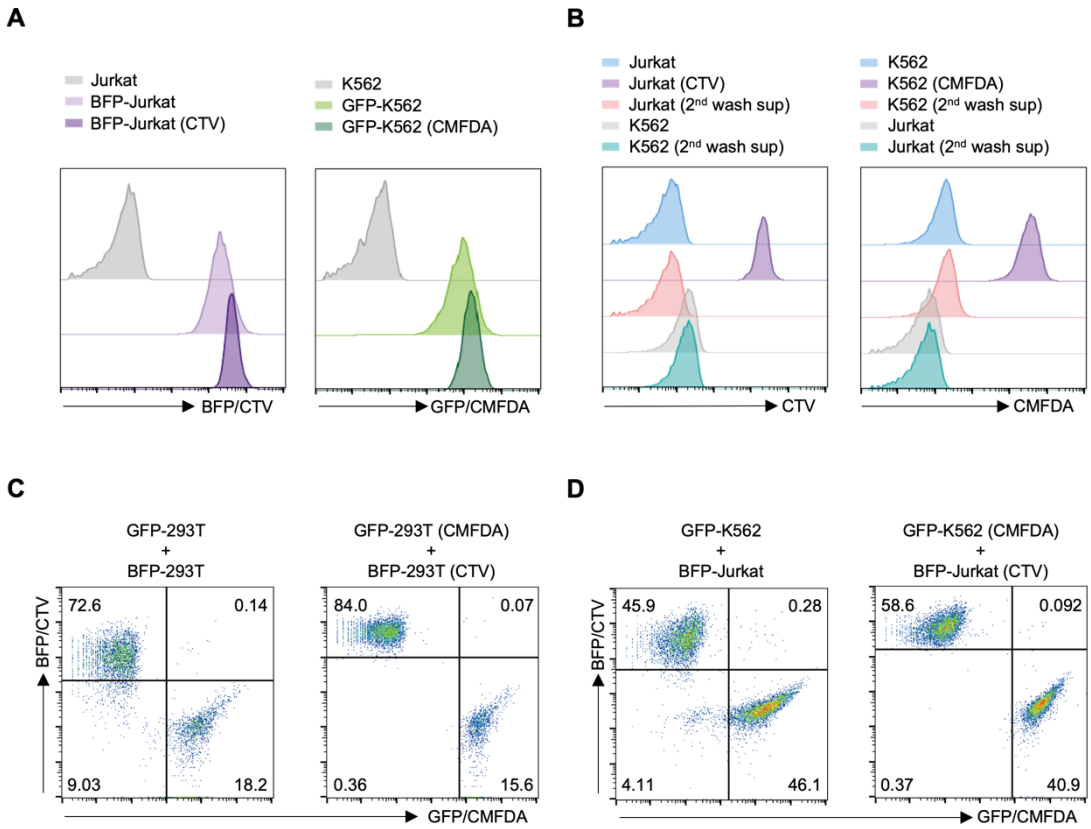
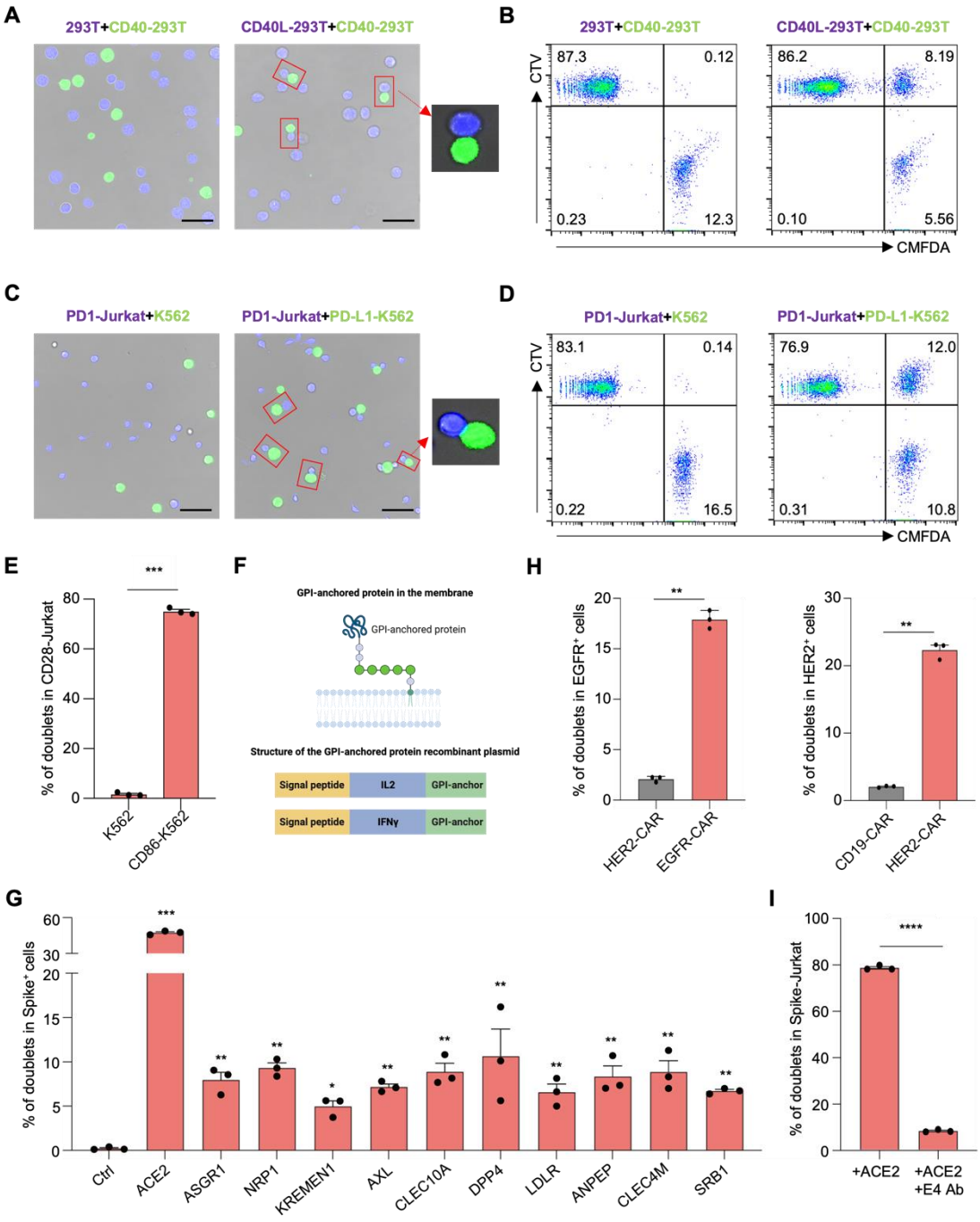
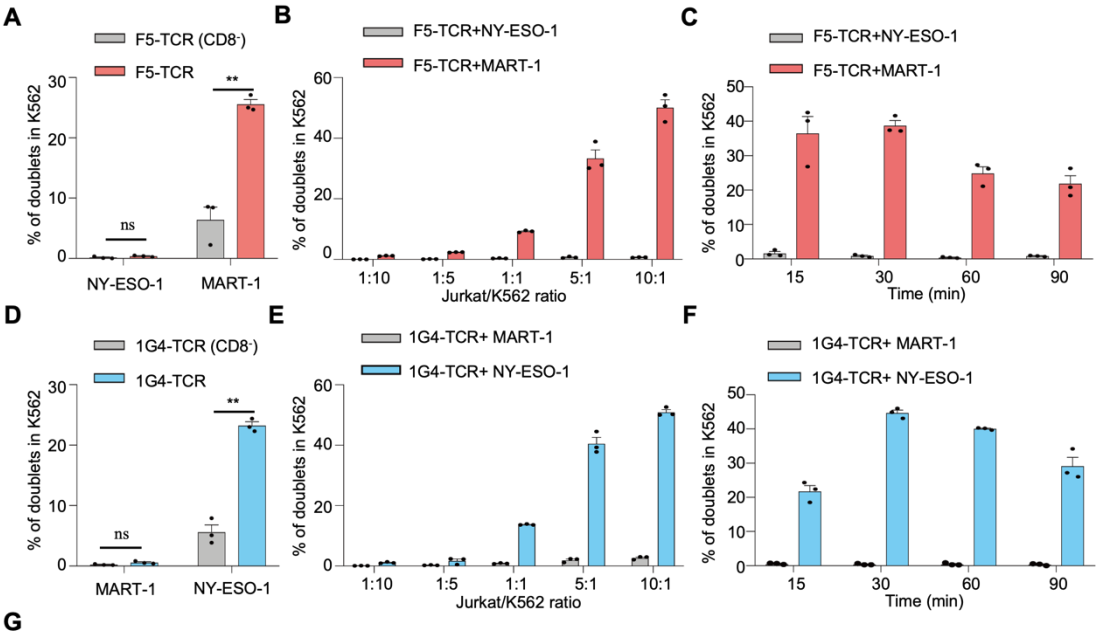


Supplementary Figure 1. Optimization of cell tracing method for minimizing signal-to-noise ratio of DoubletSeeker. (**A**) Representative image shows the background fluorescence in Jurkat, BFP-Jurkat and CTV-labeled BFP-Jurkat cells after 405 nm excitation, and in K562, GFP-K562 and CMFDA-labeled GFP-K562 cells after 488 nm excitation. (**B**) Fluorescence histogram of CTV and CMFDA in Jurkat and K562 cells, with or without staining with the secondary washing supernatant (2^{nd} wash sup) from cells following the indicated fluorescent dye staining. CTV-labeled Jurkat cells and CMFDA-labeled K562 cells served as positive controls for CTV and CMFDA fluorescence, respectively. (**C** and **D**) Two-color contour plots showing the non-specific cell doublets in indicated co-incubation groups. CTV: CellTrace^{TM} Violet, CMFDA: CellTrace^{TM} CMFDA, BFP-Jurkat (CTV): CTV-labeled BFP-Jurkat cells, GFP-K562 (CMFDA): CMFDA-labeled GFP-K562 cells, Jurkat (CTV): CTV-labeled Jurkat cells, K562 (CMFDA): CMFDA-labeled K562 cells, GFP-293T (CMFDA): CMFDA-labeled GFP-293T cells, BFP-293T (CTV): CTV-labeled BFP-293T cells. Data are representative of three independent experiments.



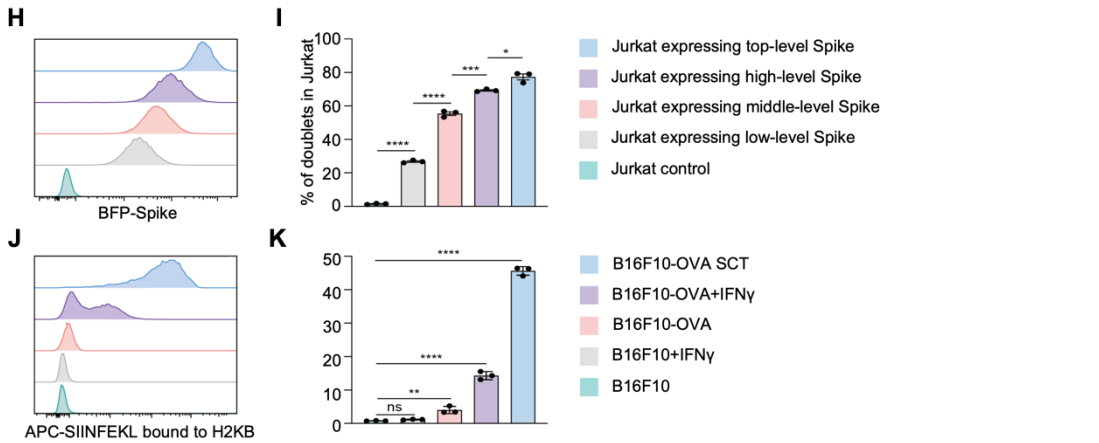
Supplementary Figure 2. DoubletSeeker's ability to decipher specific interactions between ligands and receptors for the indicated molecules. (**A** to **D**) Representative of doublets in CD40-293T (CTV-labeled, blue) co-incubated with control 293T or CD40L-293T cells (CMFDA-labeled, green) (A, B), as well as PD1-Jurkat cells (CTV-labeled, blue) co-incubated with control K562 cells or PD-L1-K562 cells (CMFDA-labeled, green) (C, D), analyzed by confocal microscopy (A, C) and flow cytometry (B, D). Red rectangle boxes mark the presence of doublets. Scale bars, 50 μm. (**E**) Formation of doublets was tested in CD28-expressing-Jurkat cells after co-incubation with either control K562 cells or CD86-expressing-K562 cells. (**F**) Schematic illustration depicts the GPI-anchored protein attached to the cell membrane and

provides an overview of the plasmid structure. (**G**) Formation of doublets after co-incubation of the cells expression Spike protein of SARS-CoV-2 (CTV-labeled) with cells expression different known host cell receptors (CMFDA-labeled). (**H**) Formation of doublets was tested between EGFR-CAR/EGFR and HER2-CAR/HER2. (**I**) Bar plots showing the percentage of doublets among Jurkat-Spike cells co-cultured with K562-ACE2 cells in the presence of SARS-CoV-2 neutralizing antibody E4 (E4 Ab). CTV: CellTrace^{TM} Violet, CMFDA: CellTrace^{TM} CMFDA. Data are represented as the means ± SEM. n=3. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001(unpaired two-tailed Student's t-test). Data are representative of three independent experiments (A to D).



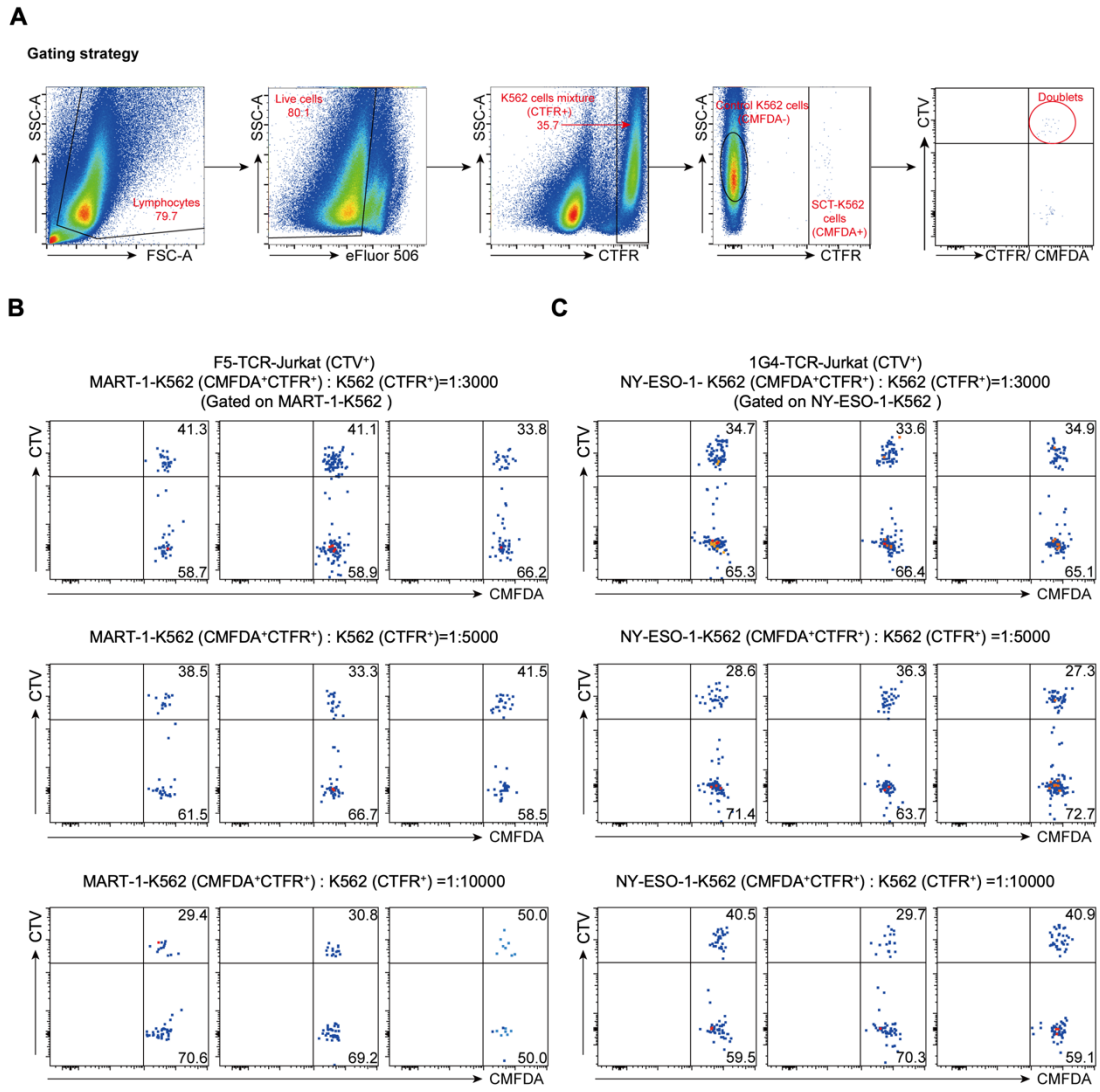
G

Ligand	Receptor	Affinity (nM)	% of doublets	References
CD19	CD19-CAR	0.17	54.77	EBioMedicine. 2020;58:102931
Spike	E4 antibody	0.82	67.17	Cell Biosci. 2022;12(1):63
IL-2	IL-2R	10.00	32.40	Math Med Biol. 2018;35(1):79-119
IFNγ	IFNγR	10.00	5.28	J Interferon Cytokine Res. 2014;34(1):41-51
Spike	ACE2	12.40	47.47	Cell Res. 2022;32(1):24-37
Spike	CLEC4M	16.00	8.87	Cell Res. 2022;32(1):24-37
Spike	KREMEN1	19.30	4.97	Cell Res. 2022;32(1):24-37
CD40L	CD40	20.00	60.27	Arthritis Res Ther. 2021;23(1):5
EGFR	EGFR-CAR	40.00	17.90	MAbs. 2020;12(1):1688616
Spike	ASGR1	94.80	7.96	Cell Res. 2022;32(1):24-37
Spike	NRP1	166.20	9.32	Pain. 2021;162(1):243-252
Spike	AXL	882.00	7.18	Sci Adv. 2023;9(2):eade0869
CD28	CD80	4000.00	31.63	Front Immunol. 2016; 7: 24
NY-ESO$_{SLLMWITQV}$-A2	1G4-TCR	4300.00	45.53	Biotechniques. 2017;62(3):123-130
MART-1$_{ELAGIGILTV}$-A2	F5-TCR	5400.00	34.77	Biotechniques. 2017;62(3):123-130
PD-L1	PD-1	8200.00	51.30	Proc Natl Acad Sci. 2015;112(47):E6506-14
MART-1$_{ELAGIGILTV}$-A2	M1W-TCR	15000.00	23.30	Biotechniques. 2017;62(3):123-130
CD28	CD86	20000.00	75.10	Front Immunol. 2016; 7: 24
Spike	CLEC10A	32000.00	8.88	Chem Soc Rev. 2023; 52(11): 3663–3740
MART-1$_{EAAGIGILTV}$-A2	F5-TCR	37000.00	15.97	Mol Ther. 2019;27(2):300-313



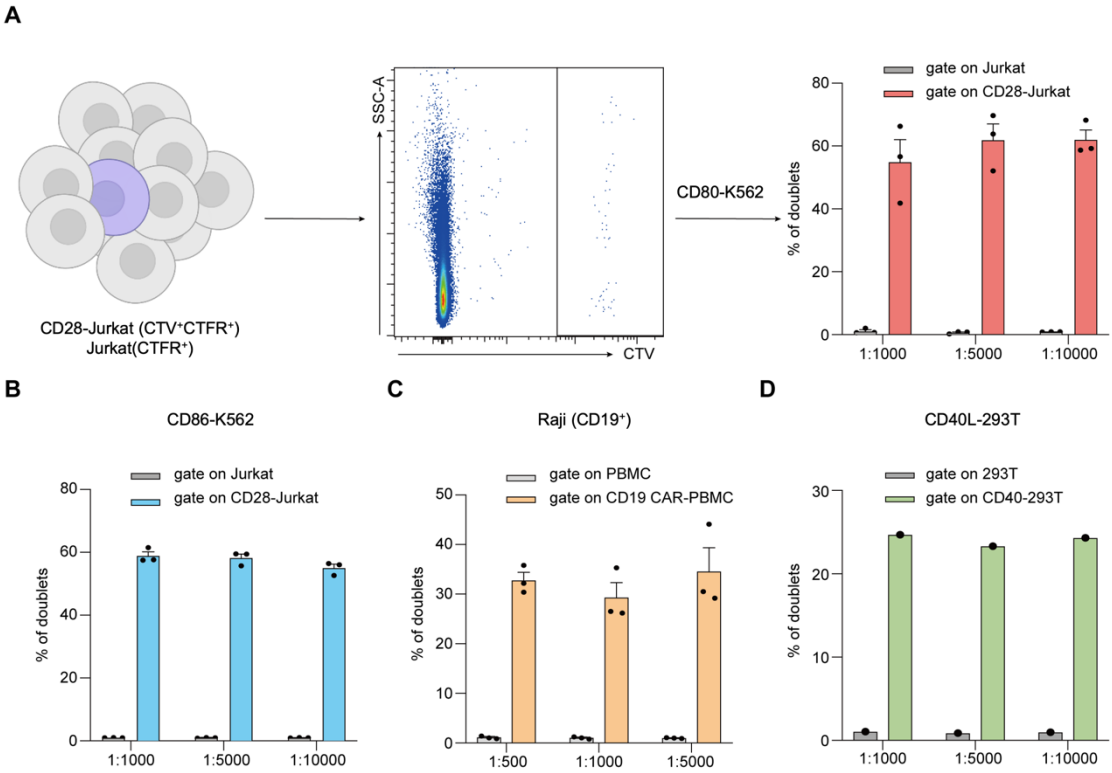
Supplementary Figure 3. Exploration of optimal experimental conditions for DoubletSeeker in the identification of TCR-pMHC interactions. (**A** and **B**) Comparison of doublets formation in MART-1-K562 or NY-ESO-1-K562 cells after co-incubation with $CD8^+$ or $CD8^-$ (CD8 non-expressed) F5-TCR-Jurkat, or 1G4-TCR-Jurkat cells (TCR-expressing cells: SCT-expressing cells=1:1). (**C** to **F**) Comparison of the percentage of doublets in MART-1-K562 or NY-ESO-1-K562 cells after co-

incubation with Jurkat cells expressing cognate or noncognate TCRs at different ratios (TCR-expressing cells: SCT-expressing cells=1:10, 1:5, 1:1, 5:1, 10:1) (C, D) or at different times (15, 30, 60, 90 min) (E, F). (**G**) List of affinities between ligands and corresponding receptors. (**H**) Representative histogram showing Jurkat-Spike cells with varying levels of Spike expression. (**I**) Bar plots showing the percentage of doublets among Jurkat-Spike cells from (H) after co-culturing with K562-ACE2 cells. (**J**) Flow cytometric analysis of SIINFEL-H2KB expression on B16F10 cells and B16F10-OVA cells with or without IFNγ pre-treatment. B16F10-OVA: B16F10 cells express OVA protein, B16F10-OVA SCT: B16F10 cells express OVA-derived SIINFEKL epitope-H2KB SCT. (**K**) Percentage of doublets among various B16F10 cells from (J) after co-culture with primary OT1 T cells. Data are represented as the means ± SEM. n=3. $^{**}P < 0.01$ and ns indicates no significance (unpaired two-tailed Student's t-test).

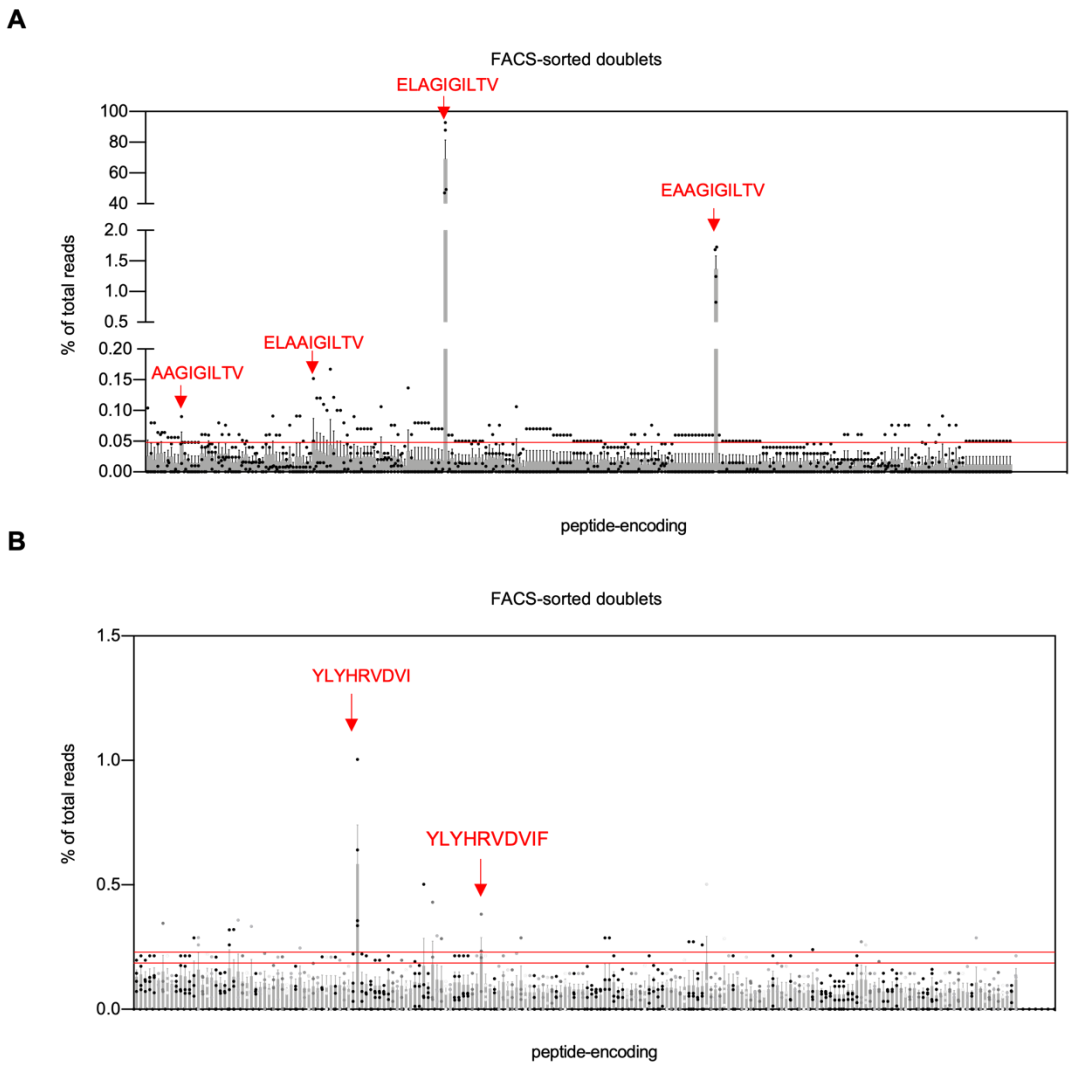


Supplementary Figure 4. Flow cytometry plots for proof-of-concept screening. (**A**) Flow cytometry gating strategy for proof-of-concept screening. (**B**) Flow cytometry plots for doublets identification in a mixture containing cognate MART-1-K562 (B) or NY-ESO-1-K562 (C) cells with noncognate K562 cells at different ratios (1:3000,

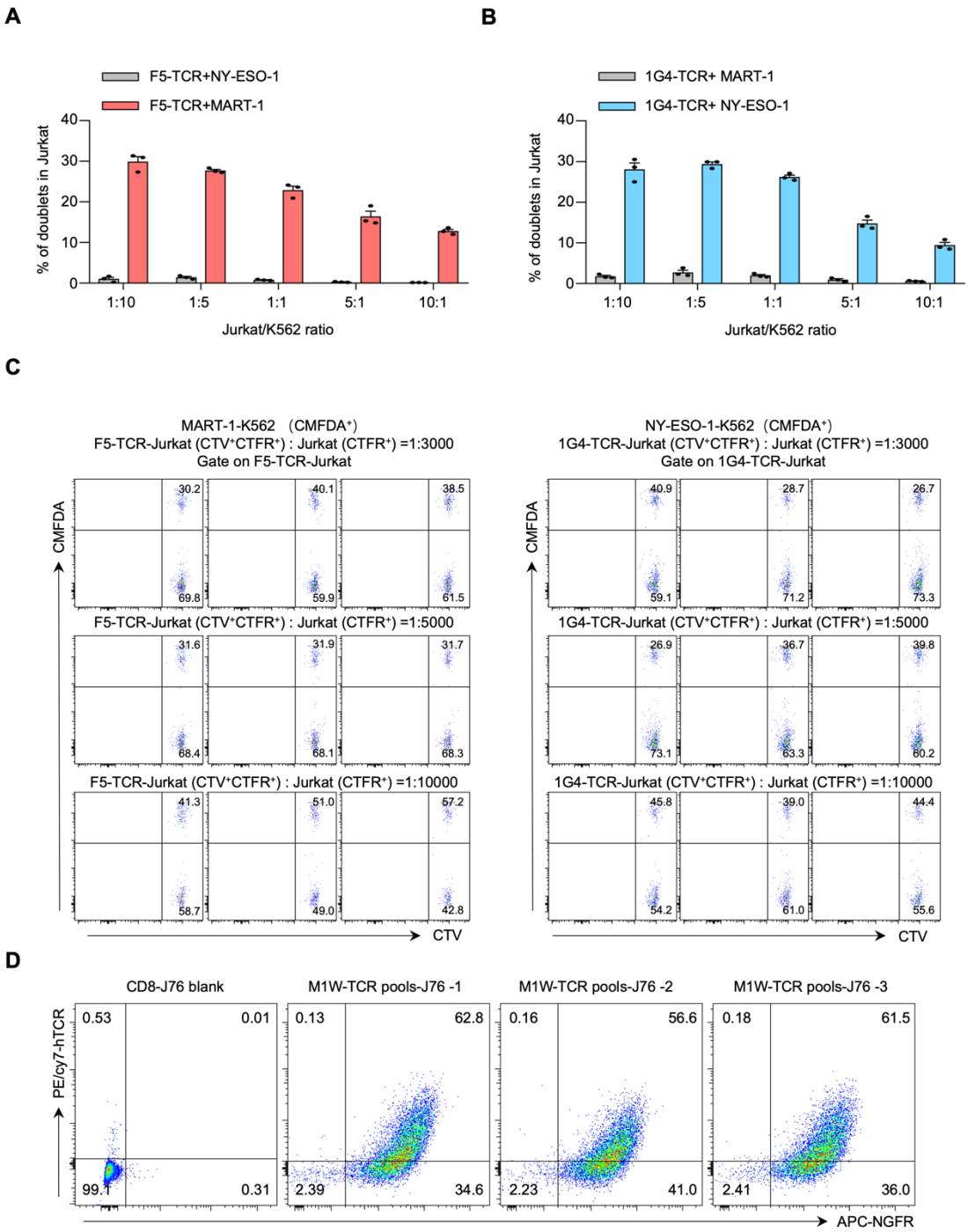
1:5000, 1:10000), co-incubated with either F5-TCR-Jurkat cells (B) or 1G4-TCR-Jurkat cells (C). MART-1-K562 and NY-ESO-1-K562 cells were labeled with CMFDA and CTFR, whereas K562 cells were labeled with CTFR. TCR-Jurkat cells were labeled with CTV. CTV: CellTrace™ Violet, CMFDA: CellTrace™ CMFDA, CTFR: CellTrace™ Far-red. *n*=3.



Supplementary Figure 5. Specificity and sensitivity of DoubletSeeker in capturing specific ligand-receptor interactions. (**A**) Schematic and representative Flow cytometry plots for doublets identification in a mixture containing CD28-Jurkat cells with control Jurkat cells at different ratios, co-incubated with CD80-K562 cells. The percentage quantification of doublets in each Jurkat cells are presented on the right. (**B** to **D**) Quantification of the percentage of doublets in each cell population from a mixture containing cells expressing the target protein (CD28, CD19-CAR, and CD40) with control cells at different ratios, following co-incubation with cells expressing the specific ligand (CD86, CD19 and CD40L). Data are represented as the means ± SEM. *n*=1 (D) or 3 (A to C). Data in are representative of three independent experiments (A).



Supplementary Figure 6. DoubletSeeker facilitates the screening of antigens for TCRs. (**A**) Reads of peptides in sorted doublets, obtained from the screening of F5-TCR targeted antigens in the A2-restricted SCT library. (**B**) Reads of peptides in sorted doublets obtained from the screening of Neo-TCR targeted antigens in the neoepitope SCT library. Data are represented as the means ± SEM. *n*=4.



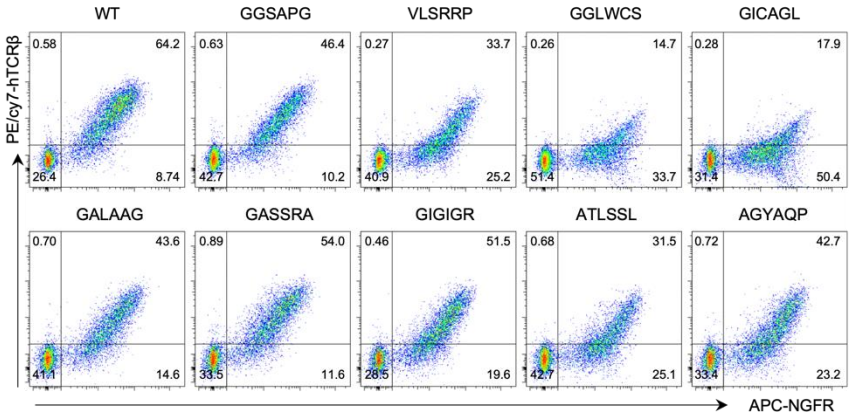
Supplementary Figure 7. Identification of TCRs specific to a given antigen by DoubletSeeker, and the construction of the M1W-TCR pools-J76 library. (**A** and **B**) Comparison of the percentage of doublets in F5-TCR-Jurkat (A) or 1G4-TCR-Jurkat (B) cells after co-incubation with either K562 cells expressing cognate or noncognate antigens at different ratios (1:10, 1:5, 1:1, 5:1 and 10:1). (**C**) Flow cytometry plots illustrating doublets in cognate F5-TCR-Jurkat cells mixed with varying proportions of the noncognate Jurkat cells mixture (left) or in cognate 1G4-TCR-Jurkat cells mixed with different proportions of the noncognate Jurkat cells mixture (right), co-incubated with MART-1-K562 cells (left) or NY-ESO-1-K562 cells (right). Doublets are analyzed by gating on cognate TCR-Jurkat populations (CTV^+CTFR^+). The colors are assigned

as TCR-Jurkat cells (CTV^+CTFR^+), noncognate Jurkat cells ($CTFR^+$), SCT-K562 cells ($CMFDA^+$) and specific doublets (CTV^+CMFDA^+). (**D**) Flow cytometry plots showing the expression of TCR and the co-expressed NGFR in CD8-J76 cells transduced with the M1W-TCR pools at an MOI of 4. CTV: CellTrace™ Violet, CMFDA: CellTrace™ CMFDA, CTFR: CellTrace™ Far-red. Data are represented as the means ± SEM. *n*= 3.

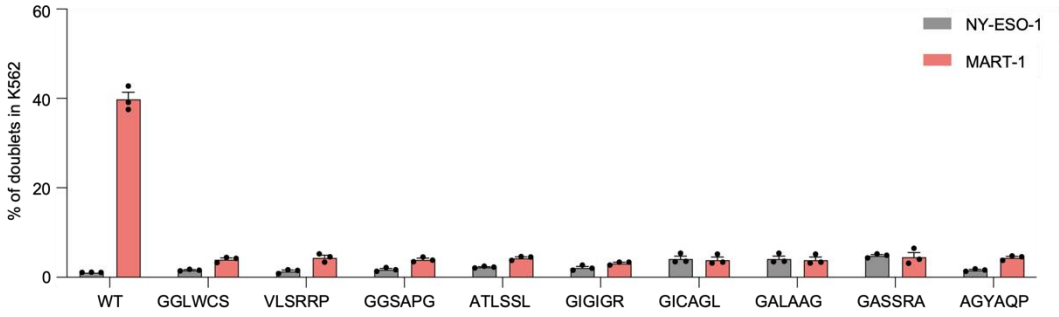
A

	Rank	Read	Percentage
CASSLVGTAGSPLHF (WT)	1	745	9.5%
CASSLGGLWCSPLHF	2	31	0.37%
CASSLVLSRRPPLHF	3	17	0.21%
CASSLGGSAPGPLHF	4	16	0.18%
CASSLATLSSLPLHF	5	15	0.17%
CASSLGIGIGRPLHF	6	11	0.14%
CASSLGICAGLPLHF	7	11	0.14%
CASSLGALAAGPLHF	8	9	0.12%
CASSLGASSRAPLHF	9	9	0.12%
CASSLAGYAQPPLHF	10	8	0.10%

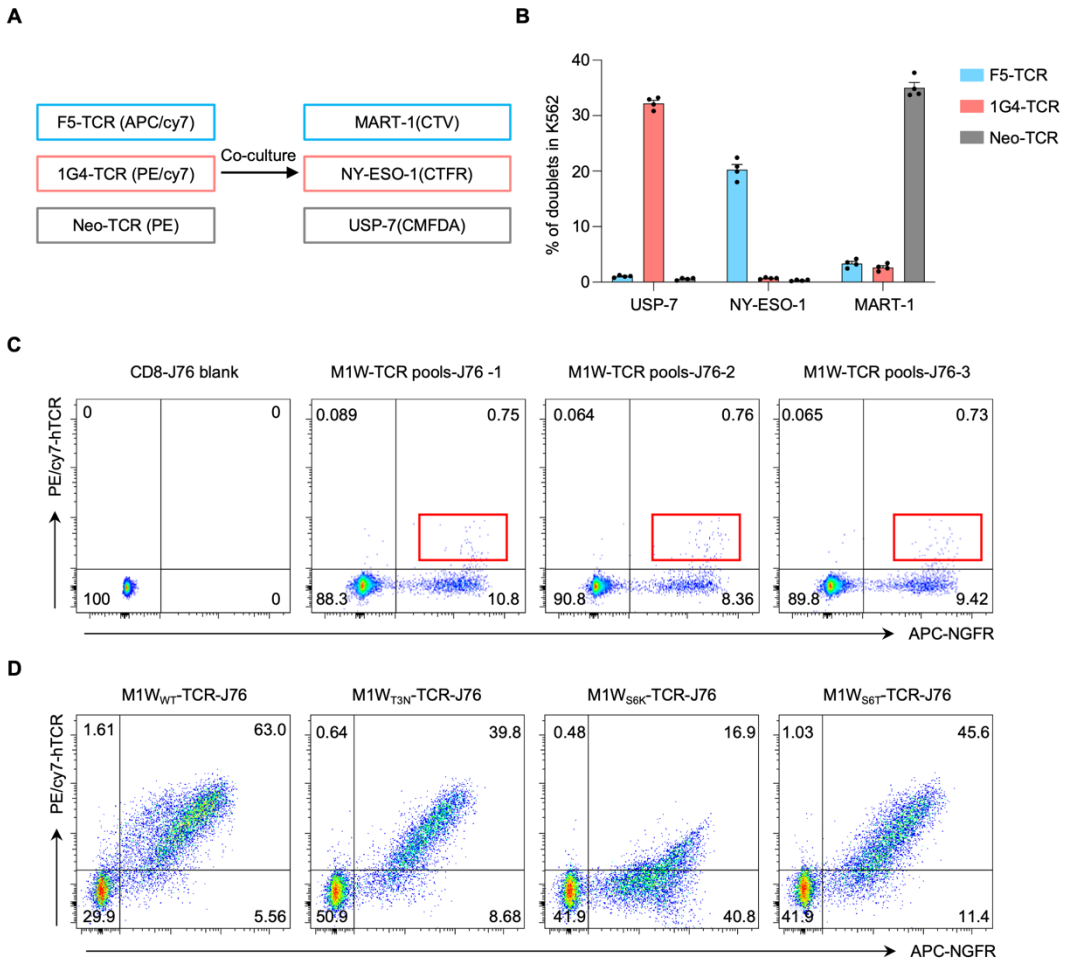
B



C



Supplementary Figure 8. Validation of the specificity of enriched M1Wmut TCRs for MART-1 ELAGIGILTV epitope via DoubletSeeker. (**A**) Read counts of the top 10 enriched TCRs in doublets sorted from the co-culture of M1W-TCR pools-J76 library with MART-1-K562 cells. (**B**) Flow cytometry plots showing the expression of TCRs and the co-expressed NGFR in CD8-J76 cells transduced with the WT M1W-TCR, as well as the enriched M1W mutant TCRs ranking from the top 2 to 9. WT M1W-TCR as a positive control. (**C**) Identification of doublets formed in MART-1-K562 or NY-ESO-1-K562 cells following co-culture with CD8-J76 cells from (B). Data are represented as the means ± SEM. *n*= 3. Data are representative of three independent experiments (B).



Supplementary Figure 9. The proof-of-concept library-on-library screening strategy and the construction of M1W-TCR pools-J76 library at an MOI of 1.
(**A**) Schematic diagram of the proof-of-concept library-on-library screening. (**B**) Antigen-specific doublets formation in the mixture of F5-TCR-Jurkat, 1G4-TCR-Jurkat, and Neo-TCR-Jurkat cells incubated with MART-1-K562, NY-ESO-1-K562 and USP7-K562 cells mixtures. (**C**) Flow cytometry plots showing the expression of TCR and the co-expressed NGFR in CD8-J76 cells transduced with the M1W-TCR pools at an MOI of 1. (**D**) Flow cytometric assessment of mutant M1W-TCRs, enriched from the "library-on-library" screening presented in Fig6.D, expression in J76 cells. CTV: CellTrace^{TM} Violet, CMFDA: CellTrace^{TM} CMFDA, CTFR: CellTrace^{TM} Far-red. Data are represented as the means ± SEM. n= 3.