Supplementary Table 1. Summary of mass spectrometry data and cell line gene expression data. Note that EPS8L2 was identified in PANC-1, but not CaSki cells, despite the RNA levels being 2x higher in CaSki, compared to PANC-1.

		PANC.1	A.375 <sup>2</sup>	SW527	CaSki	PC.3	LNCaP_clone_F GC	MCF.7	VMRC.LCD
	HLA-A <sup>1</sup>	A2/A11	A1/A2	A2/A24	A2/A3	A1/A24	A1/A2	A2/A2	A2/A2
	MAGE-A3	0.2	174.6	0.02	0.04	0.8	98.9	0.03	176.4
	EPS8L2	176.2	27.2	122.3	393.4	151.4	151.5	109.7	78.7
	MAGE-A3 pep1	No	No	No	N/D <sup>3</sup>	No	No	No	No
Identified by mass spec?	MAGE-A3 pep2	No	No	No	N/D <sup>3</sup>	No	No	No	yes
	EPS8L2	Yes	No	No	No	No	No	No	No

<sup>1</sup>TRON database

<sup>2</sup>500 nM MAGE-A3<sub>271-279</sub> peptide was loaded on 500 million cells

<sup>3</sup>A mixture of MAGE-A3<sub>271-279</sub> and MAGE-A3<sub>112-120</sub> peptides were spiked in Caski cell pellet as controls

Supplementary Table 2. Comparison of sensitivity in Jurkat cells co-cultured with peptide loaded T2, MCF7, or HEK293.

	Peptide name	Peptide seq	MAGE-A3 TCR EC50 (uM)	MAGE-A3 CAR EC50 (uM)
Т2	MAGE-A3/12 pep 1	FLWGPRALV	0.0006	0.0007
. –	MAGE-A3 pep 2	KVAELVHFL	0.01	0.06
MCF7	MAGE-A3/12 pep 1	FLWGPRALV	0.72	0.22
	MAGE-A3 pep 2	KVAELVHFL	1.88	N/A
HEK	MAGE-A3/12 pep 1	FLWGPRALV	0.047	0.011
	MAGE-A3 pep 2	KVAELVHFL	0.4	1.03

Supplementary Table 3. Examples of RNA and nuclei counts of 8 brain samples by ImageJ. Samples with high MAGE-A12 RNA counts are highlighted in yellow. These images were reexamined by eye, which revealed the high RNA counts were likely to be caused by staining artifact or over-estimation by the ImageJ algorithm. Examples are shown in Supplementary Figure 2 for areas highlighted in bright yellow. Areas in light yellow look similar to the examples and data are included in the zipped image files.

		MAGE-A12							РРІВ												
			RNA counts	5	1	luclei count	ts		RNA/nuclei	i	Average probe/nuclei	RNA counts Nuclei counts			5	Probe/nuclei			Average probe/nuclei		
		area 1	area 2	area 3	area 1	area 2	area 3	area 1	area 2	area 3		area 1	area 2	area 3	area 1	area 2	area 3	area 1	area 2	area 3	
	D006	45	12	51	250	167	362	0.18	0.07	0.14	0.13	2362	1098	1433	349	302	346	6.77	3.64	4.14	4.85
	D009	6	12	63	828	818	939	0.01	0.01	0.07	0.03	4645	1952	311	895	653	750	5.19	2.99	0.41	2.86
	D011	8	36	81	1219	1005	999	0.01	0.04	0.08	0.04	4481	5010	4977	1718	1634	1207	2.61	3.07	4.12	3.27
20200000 Imaga I	D007	28	7	32	41	63	137	0.68	0.11	0.23	0.34	966	255	781	95	57	86	10.17	4.47	9.08	7.91
20200909 Image J	D025B	26	26	19	101	78	86	0.26	0.33	0.22	0.27	213	331	399	133	169	99	1.60	1.96	4.03	2.53
	D026	23	9	7	124	133	126	0.19	0.07	0.06	0.10	42	505	527	119	85	95	0.35	5.94	5.55	3.95
	D027A	4	65	8	77	82	82	0.05	0.79	0.10	0.31	94	80	118	87	67	81	1.08	1.19	1.46	1.24
	D016B	2	54	3	40	43	31	0.05	1.26	0.10	0.47	1	4	1	39	43	36	0.03	0.09	0.03	0.05
	D006	20	12	41	226	131	273	0.09	0.09	0.15	0.11	2770	1314	1691	341	223	313	8.12	5.89	5.40	6.47
	D009	37	38	3	165	521	211	0.22	0.07	0.01	0.10	5060	1671	567	537	560	548	9.42	2.98	1.03	4.48
	D011	31	27	123	25	138	75	1.24	0.20	1.64	1.03	4282	4358	4540	692	685	581	6.19	6.36	7.81	6.79
2010 Imaga I	D007	10	5	0	48	52	129	0.21	0.10	0.00	0.10	499	118	266	75	52	60	6.65	2.27	4.43	4.45
2019 Image J	D025B	19	2	14	104	75	84	0.18	0.03	0.17	0.13	165	268	337	125	153	90	1.32	1.75	3.74	2.27
	D026	6	2	17	109	95	120	0.06	0.02	0.14	0.07	48	325	307	117	64	74	0.41	5.08	4.15	3.21
	D027A	1	1	1	67	69	61	0.01	0.01	0.02	0.02	37	51	56	70	37	73	0.53	1.38	0.77	0.89
	D016B	1	4	1	39	43	36	0.03	0.09	0.03	0.05	152	153	139	34	47	28	4.47	3.26	4.96	4.23

Supplementary Figure 1. EPS8L2 and MAGE-A12 expression in the brain from GTEx database.





Adapted from GTEx

Supplementary Figure 2. Examples of images that showed high MAGE-A12 RNA counts in Supplementary Table 3. Possible true RNA probe staining dots are pointed out by red arrows. A: D011 area 1 (2 visible dots instead of 31); B: D011 area 2 (5 visible dots instead of 27); C:D011 area3 (2 visible dots instead of 123); D: D016B area 2 (5 visible dots instead of 54); the big spots are more likely to be staining artifact than specific binding of probes; E: D007 area 1 (9 visible dots instead of 28). One cell in this area possibly showed multiple dots of MAGE-A12 RNA staining. However, this is the only cell we can find from all 8 brain samples.









D.

Single instance of possible multiple MAGE-A12-staining dots in one cell



Supplementary Figure 3. Overview of MAGE-A3 pep2 primary and binder optimization FACS sorting strategy and representative scatter plots.



## **Binder optimization library**



Supplementary Figure 4. MAGE-A3 CAR and TCR HLA-A\*02 dependent cytotoxicity. A. Cytotoxicity of engineered primary T cells against HCT116 WT (MAGE-A3+/HLA-A\*02+) and HCT116 A\*02 knockout cells at high (27:1) E:T ratio. B. HLA-A\*02 surface expression by flow cytometry; C. Emin and E0 comparison in T2/Jurkat assay between CT138 and C1511, indicating C1511 has significantly higher tonic/non-specific activity; D. C1511 showed the highest CD69 expression among MAGE-A3 CARs and TCRs without antigen challenge, indicating these cells are tonically activated. Emin: Jurkat activity with T2 cells but no added peptide; E0: Jurkat activity with no T2 cells.

Α.



## **Tonic signaling in Jurkat assay**



**E0** 

![](_page_11_Figure_3.jpeg)

![](_page_11_Figure_4.jpeg)

31.6%

13.7%

58.6%

pep1 TCR

C564

pep1 CAR

CT138

pep2 TCR

C1511

pep2 CAR

D.

![](_page_11_Figure_5.jpeg)

![](_page_11_Figure_6.jpeg)

**CT138** 

10<sup>3</sup>

CD69 Expression (FITC, Biolegend)

10<sup>4</sup>

105

Normalize

1616

903

3276

40

20 •

-10<sup>3</sup>

0

![](_page_11_Figure_8.jpeg)

Supplementary Table 4. Summary of EC50 mean and standard deviation from dose-response curves in Figure 2 and Figure 3. Comparisons are organized by construct and reflect differences in reported EC50 values for the indicated peptides (EC50s were determined from single dose-response curves). Mean and standard deviation were calculated from replicates of EC50s ( n>2). In Table 3 the average of relative signal or POC of each concentration was used to fit a single dose-response curve for EC50 calculation. Error bars represent standard deviation of each concentration in Figure 2 and 3. A-D: T/Jurkat assay results (curves shown in Figure 2): A. MAGE-A3<sub>271-279</sub> CAR and TCR across MAGE family peptides; B: MAGE-A3<sub>112-120</sub> TCR and CAR across MAGE family peptides; C: MAGE-A3<sub>271-279</sub> CAR and TCR against non-MAGE family peptides; D: Significance tests of selectivity of MAGE-A<sub>3112-120</sub> TCR and CAR against non-MAGE family peptides; E: MAGE-A3<sub>112-120</sub> TCR and CAR against MAGE-A12 and EPS8L2 in primary T cell assays (curves shown in Figure 3).

Α.	Construct	Peptide	EC50 Mean (uM)	SD (+/-)
	CT139	A3/A12	0.001	0.001
	CT139	A1/4/8	3.566	1.500
	CT139	A2/6	1.435	0.144
	CT139	A9	56.245	61.879
	CT139	A10/11	35.438	37.593
	C564	A3/A12	1.46E-04	9.79E-05
	C564	A1/4/8	0.386	0.143
	C564	A2/6	0.387	0.015
	C564	A9	33.165	6.159
	C564	A10/11	0.587	0.230

Construct	Peptide	EC50 Mean (uM)	SD (+/-)
CT138	A3	0.002	0.002
CT138	A1	>100	N/A
CT138	A2	>100	N/A
CT138	A4	>100	N/A
CT138	A5	>100	N/A
CT138	A6	>100	N/A
CT138	A8	>100	N/A
CT138	A10	>100	N/A
CT138	A11	>100	N/A
CT138	A12	4.49E-04	2.77E-05
C1511	A3	0.028	0.004
C1511	A1	>100	N/A
C1511	A2	1.099	0.088
C1511	A4	>100	N/A
C1511	A5	1.149	0.377
C1511	A6	47.185	1.068
C1511	A8	35.560	N/A
C1511	A10	>100	N/A
C1511	A11	>100	N/A
C1511	A12	0.012	1.86E-03

Β.

Construct	Peptide	EC50 Mean (uM)	SD (+/-)
CT139	A3	0.001	0.001
CT139	MAGE H1	>100	N/A
CT139	Pan MAGE A	7.993	2.609
CT139	PCSK1Na	>100	N/A
CT139	EPS8L2	>100	N/A
C564	A3	1.46E-04	9.79E-05
C564	MAGE H1	12.043	5.030
C564	Pan MAGE A	0.185	0.010
C564	PCSK1Na	0.143	0.001
C564	EPS8L2	>100	N/A

Construct	Peptide	EC50 Mean (uM)	SD (+/-)
CT138	A3	0.002	0.002
CT138	EPS8	>100	N/A
CT138	MAGE-F1	>100	N/A
CT138	MRVI1	>100	N/A
CT138	PPP2R1B	>100	N/A
C1511	A3	0.028	0.004
C1511	EPS8	25.780	1.739
C1511	MAGE-F1	8.840	0.652
C1511	MRVI1	16.335	5.112
C1511	PPP2R1B	>100	N/A

Construct	Peptide	EC50 Mean (uM)	SD (+/-)
CT138	A3	0.0006	0.0002
CT138	A12	0.0003	0.0001
CT138	EPS8L2	0.0057	0.0031
C1511	A3	1.3857	0.7496
C1511	A12	4.0880	1.0132
C1511	EPS8L2	>100	N/A