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Supplementary Materials for

Identification of a Titin-Derived HLA-A1–Presented Peptide as a Cross-Reactive Target for Engineered MAGE A3–Directed T Cells

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Video S1 (.avi format). Actively beating iCells.

Supplementary Material

Materials and Methods

OV79 tumor model

NOD-SCID-IL-2Rgc null (NSG, JAX stock # 005557) mice were produced and provided by the Stem Cell and Xenograft Core at the University of Pennsylvania School of Medicine, using breeders obtained from Jackson Laboratory (Bar Harbor). Mice were housed in sterile conditions using HEPA-filtered microisolators and fed with irradiated food and acidified water. Transplanted mice were treated with antibiotics (neomycin and polymyxin) for the duration of the experiment. Animals were used in accordance with a protocol reviewed and approved by the Institutional Animal Care and Use Committee. Six to eight week-old animals were utilized. For these experiments OV79 was engineered by lentivirus transduction to express luciferase in essentially 100% of cells (OV79-Luc). On day 0 individual mice were implanted with 1 x 10^6 OV79-luc by subcutaneous administration into the right flank in 100 µl 50% matrigel (BD Biosciences).

Following tumor implantation animals were apportioned into one of 4 cohorts, with 14 animals/ cohort. Seven days post-tumor implantation, each animal in cohorts 1-3 was injected with 1 x 10^7 CD3+ T cells prepared as described above in a final volume of 50 µl PBS. Cohorts 1 and 2 received T cells transduced to express the affinity enhanced a3a (34.8% transduced) and wild-type (28% transduced) receptor respectively, while animals in cohort 3 received non-modified but ex-vivo expanded T cells. Cohort 4 animals received no T cells. Animals were monitored weekly for tumor growth by caliper measurement and twice monthly by bioluminescence imaging. Tumor measurements (length x width) were conducted weekly using caliper measurements. Tumor volume was determined according to the formula: tumor volume (mm3) = (length x width2)/2.

Anesthetized animals were imaged using a Xenogen Spectrum system and Living Image v3.2 software. For imaging, animals, weighed on the day of imaging, were given an intraperitoneal injection of 10 mg/kg body weight D-luciferin (Caliper Life Sciences) re-suspended in sterile PBS at a concentration of 15 mg/mL (100 µL luciferin solution/10 g mouse body weight). Animals were imaged in groups of 5 at exactly 15 minutes post-luciferin injection and serial images collected at various exposure settings until the maximal exposure (60,000 counts) was reached. Data were analyzed with the Living Image v3.2 software using images taken with identical settings for mice of all groups at each time point. Imaging data were converted to photons/second/cm^2/steradian to

normalize each image for exposure time, f-stop, binning and animal size. Statistical analyses were performed using the software Prism. Survival curves were compared using a Log-rank test.

Video Microscopy

The time-lapse video of beating iCells was obtained using DIC optics and a 20x magnification objective mounted on a Zeiss 200M inverted microscope. The images were taken using MetaMorph 7.7.4.0 (Molecular Devices) stream acquisition every 2 ms and the video time frame was 5 s.

Q-RT-PCR, Mass Spectrometry and IFNγ ELISpot assays were performed as described in the main text.

Figures and Tables

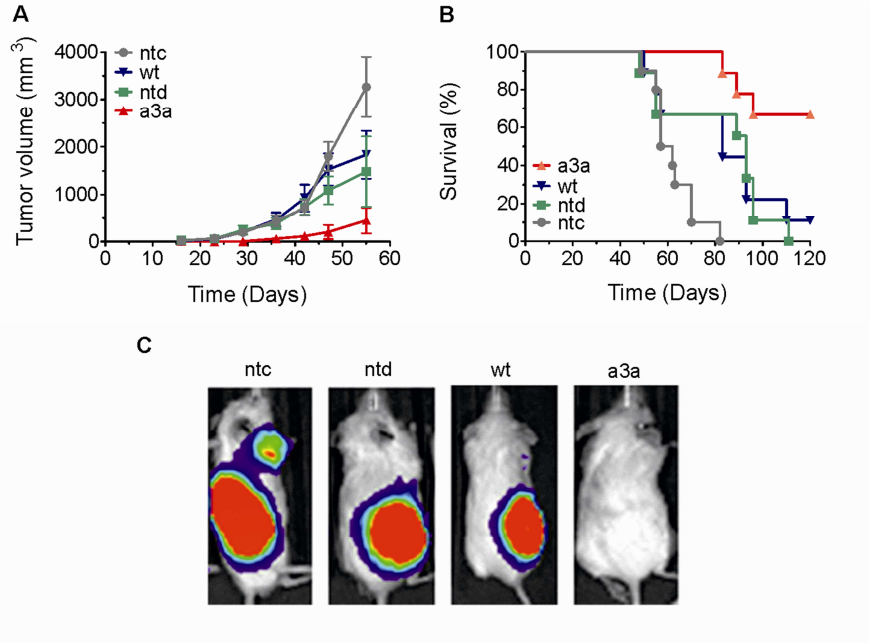

Fig. S1. Efficacy of a3a-engineered T cells in a mouse model of ovarian cancer. In each case ntc indicates no T cells, ntd indicates non-transduced cells and wt indicates T cells transduced with the non-modified TCR. (**A**) Average tumor volume measurements for each cohort, +/- SD (**B**) Kaplan-Meyer survival plots. Mice treated with T cells engineered to express the a3a receptor demonstrated a statistically significant survival rate compared to the non-transduced cells ($p = 0.005$ (Log-rank test, $n = 18$)). Injection of non-modified T cells resulted in a delay in tumor growth and a non-statistically significant ($p=0.02$ (Log-rank test, $n = 19$)) increase in median survival to 93 days compared to no T cell injection, presumably reflecting the consequence of alloreactivity of the infused T cells against the OV79 cells. While the impact of injecting T cells expressing the wild type MAGE A3 receptor was indistinguishable from that of the non-modified T cells, a3a-engineered T cells resulted in a reduction in tumor growth and survival benefit, with greater than 50% of the animals alive for this cohort at the end of the 120 day period. (**C**) Representative bioluminescence imaging data from the day 62 time-point.

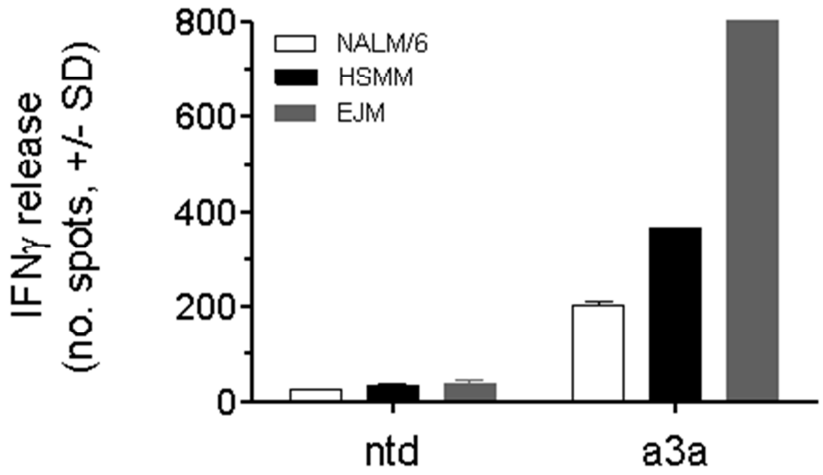

Fig. S2. Activation of a3a-engineered T cells by differentiated human skeletal muscle myoblasts (HSMM) and B cell lymphoma NALM/6 cells. Activation was determined by IFNγ ELISpot. Data represent the average of three independent measurements +/- SD. EJM cells were used as a positive control for T cell activation.

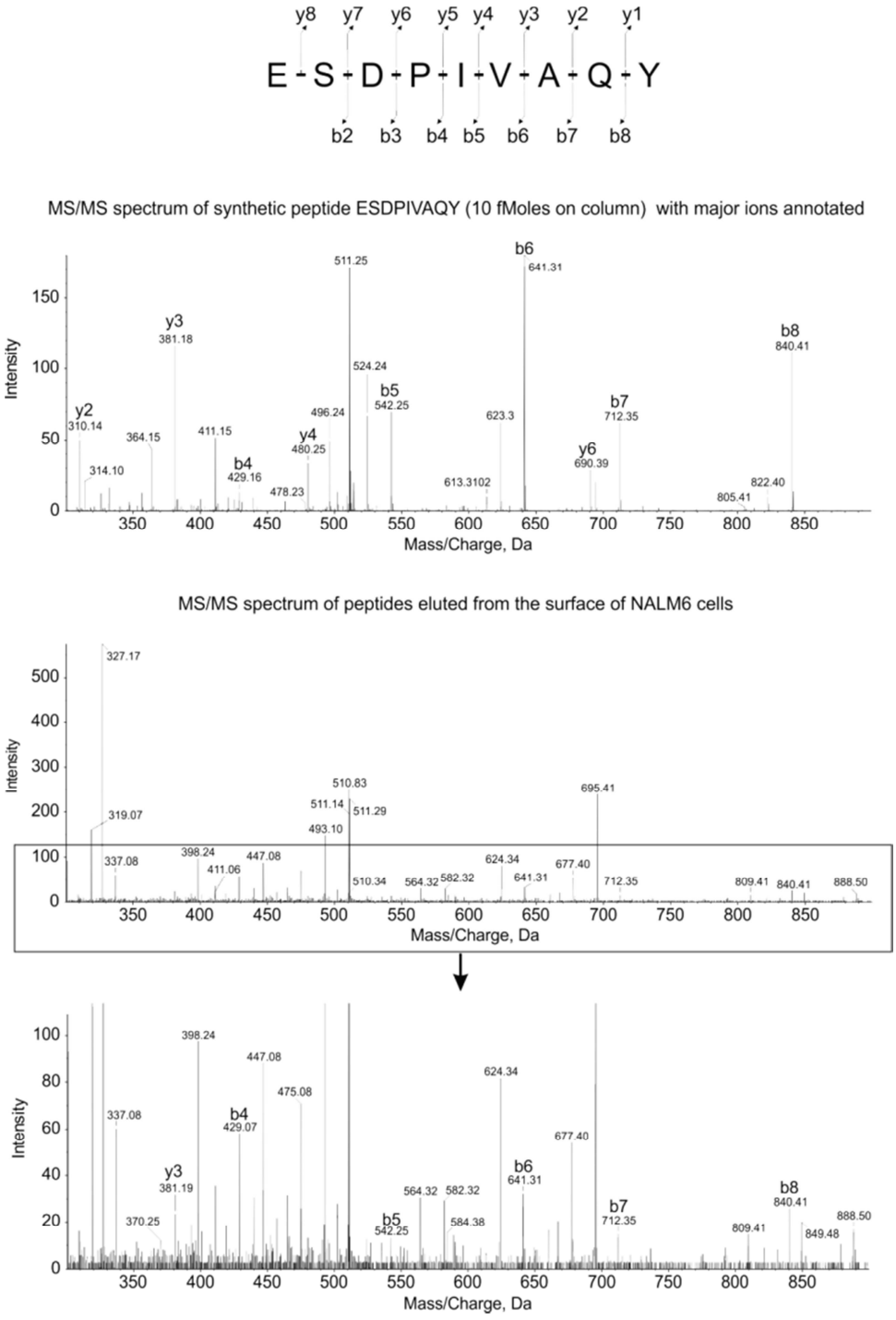

Fig. S3. Mass spectrometry fragmentation of Titin peptide. The sequence of the Titin peptide is shown at the top, with the predicted fragments indicated and the spectrum of the synthetic Titin peptide shown immediately below. Analysis of the peptides from NALM/6 cells is shown in the bottom two panels, with the lower panel showing the boxed area in greater detail. The relevant fragments of the Titin peptide are indicated.

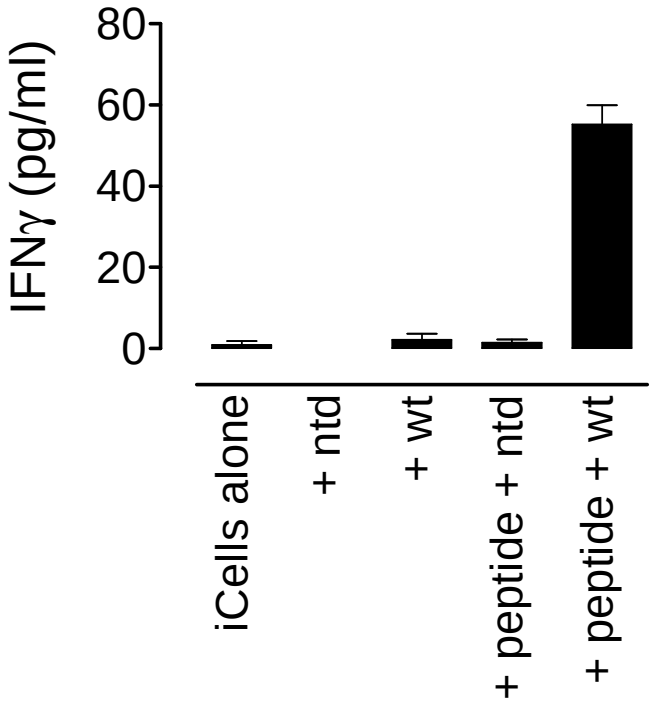

Fig. S4. T cells engineered with wild-type TCR respond to iCells pulsed with Titin peptide. iCells were transduced with HLA-A1 and incubated in the presence or absence of T cells and/or 10 uM Titin peptide as indicated. The IFNy response was measured on the Luminex platform. Non-transduced T cells (ntd) were used as a negative control. Data represent the average of three independent measurements +/- SD.

Cell line	Cell type	HLA-A*01	Reference *	MAGE A3		MAGE A6		MAGE B18	
			Ct	Ct	RQ	Ct	RQ	Ct	RQ
A375	melanoma	positive	18.9	24.3	1.000	26.4	1.000	33.1	1.000
HCT-116	colorectal carcinoma	positive	21.4	29.7	0.344	28.7	1.085	-	-
OV79	ovarian carcinoma	positive	15.1	28.3	0.004	30.8	0.004	37.8	0.003
Mel526	melanoma	negative	16.9	26.2	0.224	25.7	0.475	32.1	0.525
HEP2	normal hepatocytes	positive	15.5	-	-	-	-	-	-
M108	mesothelioma	positive	16.6	-	-	-	-	37.8	0.009
EJM	multiple myeloma	positive	23.7**	25.4	0.54	28.47	0.312	37.0	0.101
colo205	colorectal carcinoma	positive	20.8	-	-	-	-	-	-

*β-Actin
**Gus-B
- no signal detected

Table S1. qRT-PCR analysis of MAGE A3/A6/B18 expression in the tumor cell lines used in this study

Antigen	Sequence									a3a TCR cellular activity
MAGE-A3	E	V	D	P	I	G	H	L	Y	Yes
MAGE-A6	E	V	D	P	I	G	H	V	Y	Yes
MAGE-B18	E	V	D	P	I	R	H	Y	Y	Yes, low
MAGE-A1	E	A	D	P	T	G	H	S	Y	×
MAGE-A2	E	V	V	P	I	S	H	L	Y	×
MAGE-A4	E	V	D	P	T	S	N	T	Y	×
MAGE-A8/9	E	V	D	P	A	G	H	S	Y	×
MAGE-A10	E	V	D	P	T	G	H	S	F	×
MAGE-A11	E	V	D	P	T	S	H	S	Y	×
MAGE-A12	E	V	V	R	I	G	H	L	Y	×
MAGE-B1	E	D	N	P	S	G	H	T	Y	×
MAGE-B2	K	V	N	P	N	G	H	T	Y	×
MAGE-B3	K	V	D	S	T	K	D	S	Y	×
MAGE-B4	E	V	N	P	T	T	H	S	Y	×
MAGE-B5	E	V	N	P	T	C	H	L	Y	×
MAGE-B6	E	M	D	S	S	G	E	S	Y	×
MAGE-B10	E	V	E	P	N	K	H	I	Y	×
MAGE-B16	E	V	D	P	T	T	H	C	Y	×
MAGE-B17	E	M	D	P	S	R	Q	S	Y	×
MAGE-C1a	E	V	D	P	D	D	S	Y	VF	×
MAGE-C1b	E	V	D	P	D	D	S	Y	V	×
MAGE-C2a	E	V	G	P	D	H	F	C	VF	×
MAGE-C2b	E	V	G	P	D	H	F	C	V	×
MAGE-C3a	E	V	D	P	D	H	F	Y	VF	×
MAGE-C3b	E	V	D	P	D	H	F	Y	V	×
MAGE-D1/4	E	I	D	K	E	E	H	L	Y	×
MAGE-D2	E	I	D	K	N	D	H	L	Y	×
MAGE-E1	E	L	D	P	E	A	H	T	Y	×
MAGE-E2	E	V	D	T	S	E	H	I	Y	×
MAGE-F1	Q	F	D	R	K	H	H	T	Y	×
MAGE-G1	E	L	E	P	K	S	N	T	Y	×
MAGE-G1b	E	L	E	P	K	S	H	S	Y	×
MAGE-L2	E	I	D	T	K	N	H	A	Y	×

Table S2. Alternative MAGE antigens tested for cross-reactivity with a3a-engineered T cells. T cell activity was assessed by IFN-γ ELISpot

Sample	Reference*	MAGE A3		MAGE A6		MAGE B18	
	Ct	Ct	RQ	Ct	RQ	Ct	RQ
A375	25.21	29.20	1.000	31.39	1.000	36.89	1.000
Patient 1, sample 1	25.60	-	-	-	-	-	-
Patient 2, sample 1	28.11	-	-	-	-	-	-
Patient 2, sample 2	27.13	-	-	-	-	-	-
Patient 2, sample 3	27.56	-	-	-	-	-	-
Normal heart 1	26.63	-	-	-	-	-	-
Normal heart 2	29.23	-	-	-	-	-	-
Normal heart 3	27.74	-	-	-	-	-	-
Normal heart 4	26.05	-	-	-	-	-	-
Normal heart 5	26.65	-	-	-	-	-	-

*PPIB (Peptidylpropyl isomerase B)
- no signal detected

Table S3. qRT-PCR analysis of MAGE A3/A6/B18 expression patient heart tissue obtained at autopsy and from tissue obtained from normal hearts.

Cardiac tissue
Aortic Adventitial Fibroblasts
Cardiac Fibroblasts
Aortic Endothelial Cells
Cardiac Microvascular Endothelial Cells
Cardiac Myocytes
Coronary Artery Smooth Muscle Cells
Coronary Artery Endothelial Cells

Table S4. Normal cardiac cells tested for activation of a3a-engineered T cells

Cell line	Cell type	Relative expression level MAGE A3/A6*
A375 (MAGE A3 +)	Melanoma cell line	1.00
iCell cardiomyocytes	Electrically active cardiac cells	0.00
NALM/6	B cell lymphoma cell line	0.00
HSMM	Human Skeletal Muscle Myoblasts	0.00

*β-Actin was used as the reference transcript for this experiment

Table S5. qRT-PCR analysis of MAGE A3 expression in iCells, NALM/6 and human skeletal muscle myoblast cells

Antigen	Sequence									a3a TCR cellular activity
MAGE-A3	E	V	D	P	I	G	H	L	Y	Yes
MAGE-A6	E	V	D	P	I	G	H	V	Y	Yes
MAGE-B18	E	V	D	P	I	R	H	Y	Y	Yes (low)
Caveolin-1	Y	V	D	S	E	G	H	L	Y	×
FGD5	E	V	G	P	I	F	H	L	Y	×
ERRFI1	N	I	D	P	I	T	M	A	Y	×
RFWD2	V	V	D	N	I	D	H	L	Y	×
DMXL2	R	V	D	P	I	G	P	L	S	×
ATF4	T	V	N	P	I	G	H	L	P	×
PZP	P	K	A	P	V	G	H	L	Y	×
LMX1A	V	G	N	P	I	D	H	L	Y	×
AOX1	P	E	D	P	I	G	H	P	I	×
ARAP3	L	A	T	L	I	G	H	L	Y	×
MARS2	A	A	P	H	I	G	H	L	Y	×
SYNGAP1	E	V	D	P	I	K	C	T	A	×
TNRC6B	S	P	D	P	I	G	H	N	P	×
COEA1	E	V	D	P	I	T	T	F	P	×
BRD4	V	F	D	P	I	G	H	F	T	×

Table S6. Peptides identified in a BLAST search and tested for cross-reactivity with a3a-engineered T cells. Activity was determined by IFNγ ELISpot

Video 1 Actively beating iCells

See separate file