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Re-examination of MAGE-A3 as a T-cell Therapeutic Target

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Summary: In 2013, an innovative MAGE-A3-directed cancer therapeutic of great potential value was terminated in the clinic because of neurotoxicity. The safety problems were hypothesized to originate from off-target T-cell receptor activity against a closely related MAGE-A12 peptide. A combination of published and new data led us to test this hypothesis with current technology. Our results call into question MAGE-A12 as the source of the neurotoxicity. Rather, the data imply that an alternative related peptide from EPS8L2 may be responsible. Given the qualities of MAGE-A3 as an onco-testis antigen widely expressed in tumors and largely absent from normal adult tissues, these findings suggest that MAGE-A3 may deserve further consideration as a cancer target. As a step in this direction, the authors isolated 2 MAGE-A3 peptide-major histocompatibility complex-directed chimeric antigen receptors, 1 targeting the same peptide as the clinical T-cell receptor. Both chimeric antigen receptors have improved selectivity over the EPS8L2 peptide that represents a significant risk for MAGE-A3-targeted therapeutics, showing that there may be other options for MAGE-A3 cell therapy.

Key Words: MAGE-A12 cross-reactivity, off-target, onco-testis antigen, EPS8L2, neurotoxicity, T-cell receptor, chimeric antigen receptor

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Off-target toxicity is one of the major risks of investigational therapies. Adoptive transfer of engineered T cells, which reached a milestone recently with the approval of 2 CD19-directed cancer therapeutics, is no different. Indeed, there are several examples of toxicities—including fatal ones—believed to be caused by the therapeutic administration of investigational T-cell therapeutics. The risk is thought to be higher with T-cell receptors (TCRs) that are modified after isolation from patients, and not used in their pristine form.[1–5] In 2 well-publicized cases, the origin of the toxicity has been ascribed to homologous peptides that bind the HLA class I allele of the on-target peptide-major histocompatibility complex (pMHC) and cross-react with the affinity-enhanced TCR used to create the therapeutic candidate.

In one of these cases, a TCR-T containing a TCR (A118T) generated by immunization of an HLA-A*02 transgenic mouse with a peptide derived from the cancer testis antigen MAGE-A3 (residues 112–120, KVAELVHFL) was terminated during phase 1 clinical study despite early signs of efficacy.[2] Three of 9 cancer patients treated with the TCR-T developed severe neurotoxicity, and though each patient's history contained complicating factors, the toxicity was classified as treatment-related, and the investigators discontinued the trial for safety reasons. Histopathology supported the view that the TCR-Ts caused T-cell infiltration in the brain.

To explore the basis for toxicity, the investigators considered a variety of possibilities, foremost among them, TCR cross-reaction with MAGE family members potentially expressed in the brain. Of these paralogs, MAGE-A12 was considered a prime suspect, as strong TCR cross-reactivity with the homologous peptide that differs by only one residue from the MAGE-A3 peptide had been noted during the original characterization of this TCR.[6] The authors conducted a thorough study of MAGE-A12 expression in the brain using a variety of methods, including 3 different types of mRNA quantification and immunohistochemistry (IHC). They concluded that MAGE-A12 cross-reaction was the best hypothesis for the cause of the neurotoxicity induced by the TCR because: (i) all MAGE family members, including *MAGE-A3*, are expressed at low or undetectable levels in the brain, with the exception of *MAGE-A12*, whose expression in brain samples is low, but detectable at the RNA level [$<\sim$1 transcripts per million (TPM)]; (ii) IHC using an anti-MAGE family antibody yielded rare but strongly positive cells in patient and control brain sections ($\sim$1/100 brain cells); and (iii) the TCR had $\sim$10× higher sensitivity to MAGE-A12$_{112–120}$, compared with the on-target MAGE-A3 pMHC.

Here we report the results of a test of the MAGE-A12 hypothesis and propose an alternative. The RNA expression data combined with the IHC experiment published by Morgan and colleagues predict that MAGE-A12 mRNA is concentrated at high levels in rare cells. We were unable to detect a family of rare high-expressing cells in well-controlled RNA fluorescence in situ hybridization (RNA FISH) experiments, evidence that does not conform with a key prediction of the hypothesis. Moreover, we propose an alternative candidate to explain the neurotoxicity: a related pMHC peptide derived from the EPS8L2 protein. Finally, we compare the TCR to 2 chimeric antigen receptors (CARs), one directed at the same MAGE-A3 pMHC, the other against a peptide common to both MAGE-A3 and -A12 (Table 1). We show that the CARs have good

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TABLE 1. Peptides and Constructs Used in This Study

Peptide Name	Position	Sequence	Constructs
MAGE-A3$_{271–279}$	271–279	FLWGPRALV	CT139 (TCR) C564 (CAR)
MAGE-A3$_{112–120}$	112–120	KVAELVHFL	CT138 (TCR) C1511 (CAR)

CAR indicates chimeric antigen receptors; TCR, T-cell receptor.

selectivity in general, and discriminate significantly better against the EPS8L2 peptide than the TCR. These results are important because they provide a potential path forward to test additional cancer therapeutics directed at the MAGE-A3 peptide known to be displayed in a large population of cancers.[7]

RESULTS

MAGE-A12 Expression is Very Low in the Brain

To add support to the data presented in Morgan and colleagues that *MAGE-A12* expression, averaged across the brain, is extremely low, we collated information from 3 high-quality public RNA-Seq databases (GTEx, Human Protein Atlas, and FANTOM5). Because of its breadth and depth, we focus here on quantification derived from GTEx.[8,9] RNA-Seq has several advantages compared with other methods of RNA quantification: (i) high sensitivity, constrained mainly by sequencing depth; (ii) high selectivity, based on highly specific DNA sequence calls of current algorithms; and (iii) linear, large dynamic range.[10] With GTEx, we confirmed the results of the previous analysis, with a median *MAGE-A12* expression level of 5.9 TPM in the testis (n = 361; Table 2[9,11,12]). According to GTEx, across multiple regions of the brain the maximum value was 0.3 TPM in basal ganglia (n > 151 samples/brain region). For comparison, *MAGE-A3* expression was 12.8 TPM in testis, with no reported transcript in any of 13 brain regions examined. Of other MAGE paralogs, the highest brain expression belonged to *MAGE-A10* (median <0.1 TPM/brain region), consistent with the evidence that the MAGE family of cancer testis antigens has very low adult normal-tissue expression outside the testis.

An average mammalian cell contains ~200,000 mRNAs/cells,[13] a neuron based on its above-average size, presumably more. If we use this average value, we expect that MAGE-A12 would be present on average at > 0.06 mRNAs/cell. From the histopathology image in figure 12 of Morgan and colleagues' study, we estimated ~1 in 100 cells stained with the MAGE antibody (6C1) used by the authors (obtained from Santa Cruz Biotechnology, Santa Cruz, CA). Therefore, the stain-positive cells should contain ~100× more MAGE-A12 mRNA, compared with their stain-negative neighbors; thus, > 6 transcripts/cell. We, therefore, designed an experiment to detect the presence of these cells using RNA FISH.

We generated and tested an RNA FISH probe set that was predicted to be selective for *MAGE-A12* over *MAGE-A3*. Because of the high sequence similarity among MAGE paralogs, it was necessary to maximize identities between the probe and MAGE-A12, and mismatches with the related off-target mRNAs (Fig. 1A; see the Materials and methods section). These probes were tested on cell lines that expressed either high (K562) or low (PC3) MAGE-A12 levels (Fig. 1B). Positive- and negative-control RNA FISH probes were also used in the hybridization. From these initial experiments, we determined that the RNA FISH technique was sufficiently sensitive at its limit to perhaps detect the very low level of MAGE-A12 mRNA present in PC3 cells (0.05 TPM), and certainly register transcripts in K562 cells[14] (87 TPM). A small number of stain dots, statistically higher than observed in the negative-control probe sections, were visible in PC3 cells (~0.1 dots/cell). In contrast, numerous dots were visible on the K562 sections, averaging 2–3 dots/cell. These results suggest that the RNA FISH technology used in these experiments has high sensitivity—potentially able to detect mRNAs at <1 transcript/cell on average—but is nonlinear. Results from the positive-control probe were consistent with this observation.

Having qualified the RNA FISH probes, we next tested brain tissue sections from 8 postmortem donors (5 normal and 3 cancer patient donors). In total, ~3.2 million brain cells were scanned by eye by 3 individual scientists (not including scientists at Advanced Cell Diagnostics). The results were further confirmed using ImageJ automated analysis software (see example scans in Supplementary Table 3, Supplemental Digital Content 1, http://links.lww.com/JIT/A595). The images with high RNA counts called by ImageJ were re-examined visually. Inspection of these images revealed RNA counts to be extremely low in these areas, indicating the ImageJ algorithm likely overestimated the number of RNA-stained puncta in some circumstances (examples shown in Supplementary Figure 2, Supplemental Digital Content 1, http://links.lww.com/JIT/A595). No expression of *MAGE-A12* was observed above the detection limit determined by the negative-control probe (Fig. 1C). We were especially vigilant for high-expressing cells with multiple dots/cell; one out of ~3 million cells was seen. Thus, we did not detect the predicted family of rare MAGE-A12 high-expressing cells in the brain, even though they would fall within the dynamic range of the RNA FISH assay. This result, together with the other RNA data, is not consistent with the hypothesis that MAGE-A12 is the underlying cause of neurotoxicity observed with the MAGE-A3 clinical TCR.

EPS8L2 Cross-reactivity is an Alternative Cause for the Observed TCR-T Neurotoxicity

We sought to obtain evidence for other possible sources of the observed neurotoxicity. Morgan and colleagues noted that their TCR also cross-reacted with a peptide (*SAAELVHFL*) from another protein, EPS8L2, although 10–100× more weakly than MAGE-A3. In the GTEx database, EPS8L2 expression was high in several tissues, including the cerebellum (64 TPM, n = 241), over 100× higher than MAGE-A12 RNA levels. RNA level correlates with protein level, which in turn correlates with the likelihood of pMHC display.[15] EPS8L2 protein is detected in brain samples documented by the Human Protein Atlas database.[16] Morgan et al stated that full-length EPS8L2 expressed exogenously did not activate the MAGE-A3 TCR. However, we detected the cross-reactive EPS8L2 peptide in a mass spectrometry experiment using a cell line (PANC-1) that expresses 176 TPM endogenous EPS8L2 (Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/JIT/A595; see the Materials and methods section). In contrast, we failed to detect this peptide in 7 other HLA-A*02$^+$ cell lines that also express EPS8L2 (range, 27–393 reads per kilobase million). These results were qualitatively similar for MAGE-A3$_{112–120}$ peptide, where the peptide was observed in only one of the 3 HLA-A*02$^+$

TABLE 2. Expression of Genes of Interest in Primary Tissue and Cell Lines Used in This Study

Genes	Testis (TPM)*	Brain Region (TPM)* All (Avg.)	Basal Ganglia†	Cerebellum†	HEK293‡	MCF7 (RPKM)§	CAPAN-2 (RPKM)§	A375 (RPKM)§	PC3 (RPKM)§	K562 (RPKM)§
MAGE-A3	12.8	ND	ND	ND	No/low	0.03	0.1	175	0.8	74
MAGE-A12	5.9	0.2	0.3	0.1	No/low	0.04	0.1	245	0.05	87
MAGE-A10	3.8	0	ND	ND	No/low	0	0.07	74	0.04	0.3
EPS8L2	13.0	12.6	5.4	64.0	No/low	109	453	27	151	12

*Highest expression region for MAGE family members; GTEx.[9]
†Highest expression region in the brain for the target gene (average of putamen and nucleus accumbens).
‡BioGPS,[11] below median of >60 cell lines on the U133A Affymetrix chip.
§TRON.[12]
ND indicates no TPM reported; TPM, transcripts per million.

lines that expressed MAGE-A3 RNA. This suggests that despite high expression, the EPS8L2 and MAGE-A3 peptides are displayed only under certain circumstances, or at the limit of detection of mass spectrometry, which is thought to be less sensitive for pMHC detection than T-cell functional assays.[17]

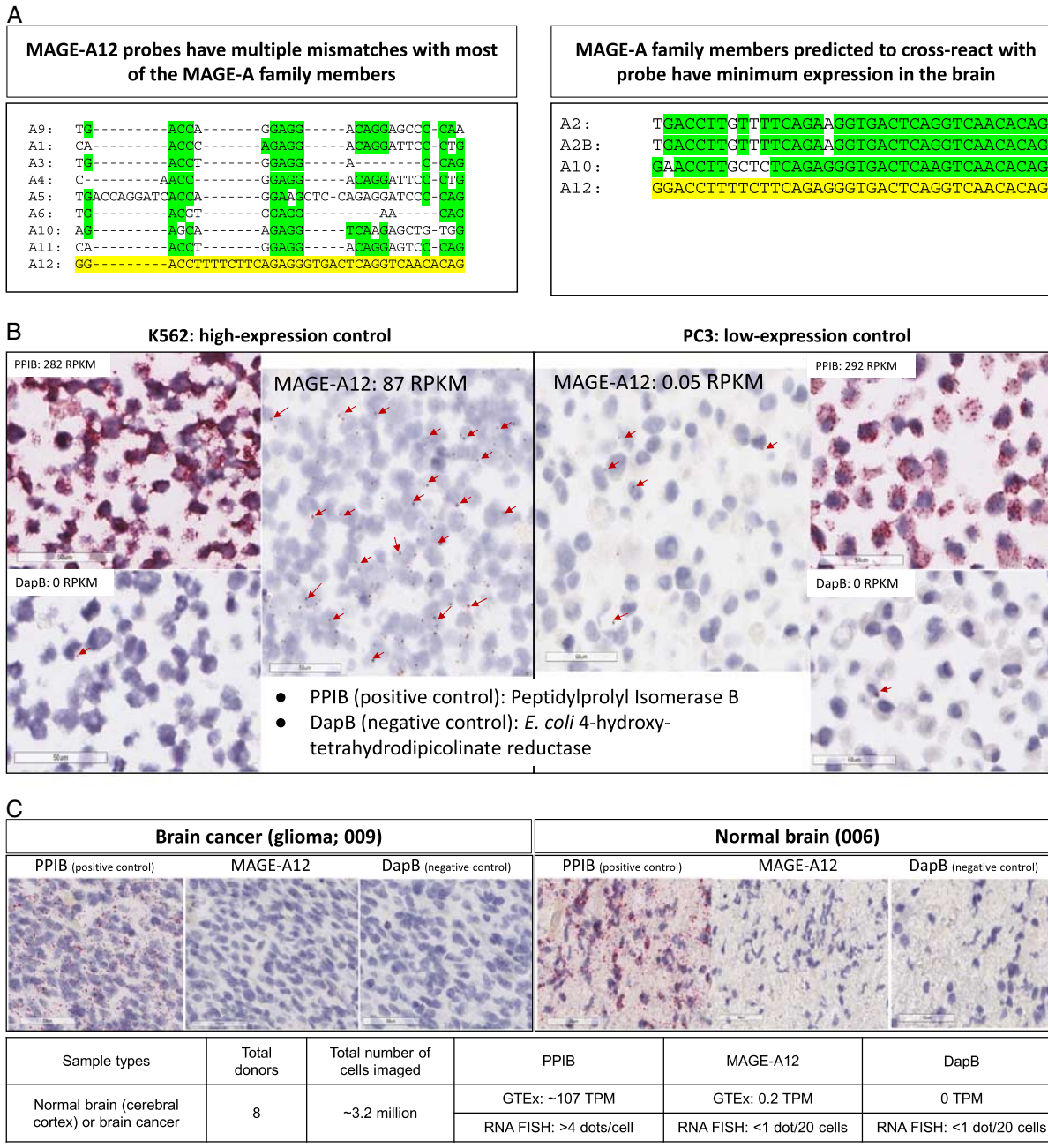
We confirmed the cross-reactivity with MAGE-$A12_{112–120}$ and $EPS8L2_{339–347}$ using the clinical TCR in Jurkat/T2 cell assays[18,19] (Fig. 2; Table 3; Supplementary Table 4, Supplemental Digital Content 1, http://links.lww.com/JIT/A595; see the Materials and methods section). Compared with the on-target MAGE-A3 peptide, the TCR was ~10× more potent against MAGE-A12 and ~500× less potent against $EPS8L2_{339–347}$.

We next tested sensitivity and selectivity of the MAGE-$A3_{112–120}$ TCR in primary human T-cell assays, using peptide-loaded HLA-A*02^+ MCF7 and HEK293 cells, as these target cell lines do not express endogenous *MAGE-A3* or *-A12* (Fig. 3; Table 2). Not only did the TCR not discriminate between MAGE-A3 and MAGE-A12 peptides but also it displayed significant cross-reactivity with EPS8L2, within ~10× of the on-target EC50. In HEK293 cells, TCR activity was right-shifted ~100× in sensitivity compared with MCF7, perhaps because peptide loading and/or killing are less efficient (Supplementary Table 2, Supplemental Digital Content 1, http://links.lww.com/JIT/A595). But again, the TCR showed significant cross-reactivity to EPS8L2, comparable with the on-target MAGE-A3 peptide. These results confirm that the TCR is sensitive to $EPS8L2_{339–347}$ particularly in primary T-cell cytotoxicity.

Lastly, we tested if the MAGE-$_{2–120}$ TCR could mediate cytotoxicity through the native expression of EPS8L2 protein. The MAGE-$A3_{112–120}$ TCR triggered the killing of cell lines that express MAGE-A3 (Figs. 4, 5; Supplementary Figure 4, Supplemental Digital Content 1, http://links.lww.com/JIT/A595). However, in HLA-A*02-expressing CAPAN-2 cells, a cell line that expresses EPS8L2 (~450 TPM) but not MAGE-A3 or -A12 (both <1 TPM), the TCR also mediated significant cytotoxicity. The use of parental CAPAN-2 that does not express HLA-A*02 demonstrated that killing was dependent on the expression of the pMHC complex. Thus, $EPS8L2_{339–347}$ has properties consistent with causing the toxicity responsible for the termination of the A118T TCR in the clinic: (i) It elicits a strong response from the TCR in carefully controlled peptide-loading experiments; (ii) It is expressed at high levels in the brain—much higher than MAGE-A12; (iii) It is detected by mass spectrometry of immune-precipitated HLA class I pMHCs isolated from an EPS8L2-expressing cell line; And, (iv) in the absence of MAGE-A3/12 endogenous expression, it provides an explanation for residual HLA-A*02-dependent cytotoxicity displayed by the TCR against cells that express EPS8L2.

MAGE-A3-directed CAR-Ts as an Option for Cell Therapy

In principle, binders based on monoclonal antibodies (mAbs) obtained through immunization or in vitro display provide an alternative to TCRs.[20] To ascertain if we could identify (scFv) binders with better functional selectivity than the clinical TCR, we screened for MAGE-A3-directed CARs with a focus on both MAGE-A12 and EPS8L2 as potential off-target peptides. We screened a mAb-display library in human cells for MAGE-A3 pMHC mAbs that bind the same target as the clinical TCR (HLA-A*02 complexed with MAGE-$A3_{112–120}$)[21,22] (Supplementary Figure 3, Supplemental Digital Content 1, http://links.lww.com/JIT/A595). To maximize the chance for selectivity, we counter-screened against off-target pMHCs, including MAGE-A12 and EPS8L2. We also screened for binders against a second MAGE-A3 pMHC presented by HLA-A*02 with identical sequence to the homologous MAGE-A12 proteins[6] (MAGE-$A3_{271–279}$/$A12_{271–279}$; FLWGPRALV). After screening for binders, we converted the mAbs to scFv CARs and tested them in Jurkat functional assays, with peptide-loaded T2 cells as a stimulus. We focused on 2 mAbs, one against each MAGE-A3 pMHC (Table 1). Both were selective against many related peptides, including peptides derived from members of the MAGE family (Fig. 2A, B). Indeed, both binders exhibited TCR-like selectivity at the level of single amino acid differences, though the A118T clinical TCR was, in general, more discriminating. This discrimination was also apparent when a panel of off-target peptides was used to compare reactivities at high concentrations. But importantly, the MAGE-$A3_{112–120}$ CAR was ~10× more selective for MAGE-A3 over MAGE-12 compared with the TCR, and at least 6× more selective over $EPS8L2_{339–347}$ in Jurkat/T2 assays, suggesting that improved selectivity against these potential off-target pMHCs is possible despite their high similarity to the on-target MAGE-$A3_{112–120}$ peptide. In primary T-cell assays, the MAGE-$A3_{112–120}$ CAR retained modest selectivity for the MAGE-$A12_{112–120}$ peptide, with no detectable activity against $EPS8L2_{339–347}$ peptide (Fig. 3). Both the MAGE-$A3_{271–279}$ TCR and CAR (C564), and the MAGE-$A3_{112–120}$ TCR, showed selective HLA-A*02-restricted



Sample types	Total donors	Total number of cells imaged	PPIB	MAGE-A12	DapB
Normal brain (cerebral cortex) or brain cancer	8	~3.2 million	GTEx: ~107 TPM	GTEx: 0.2 TPM	0 TPM
			RNA FISH: >4 dots/cell	RNA FISH: <1 dot/20 cells	RNA FISH: <1 dot/20 cells

FIGURE 1. MAGE-A12 expression in human brain samples. A, Tissue/cell RNA FISH: specific probes designed to determine the expression of MAGE-A12 in the brain and cell lines. The BaseScope probe can potentially cross-react with MAGE-A2, A2b, and A10. However, the RNA expression of MAGE-A2b and MAGE-A10 (shown in Table 2) are 0 TPM in the brain as reported in the GTEx database. There is no RNA expression (TPM value) for MAGE-A2 in the brain in GTEx. Green: identical nucleotide acid to the BaseScope probe; yellow: BaseScope probe. B, Detection of MAGE-A12 using cell lines with known MAGE-A12 expression levels. Examples of stain dots are highlighted with red arrows. RPKM values were extracted from the TRON database. C, Examples of ~3 million brain cells from 8 donors that were stained using MAGE-A12-specific probe and scanned by eye and ImageJ software. No expression of MAGE-A12 higher than the negative control was detected. Only a single cell with multiple dots of MAGE-A12 RNA staining was observed (see Supplementary Fig. 2D, Supplemental Digital Content 1, http://links.lww.com/JIT/A595). Cells with MAGE-A12 expression level predicted from the combination of RNA-Seq databases and Morgan and colleagues (2–20 TPM in the brain) are expected to be well within the dynamic range of the assay. RNA FISH, RNA fluorescence in situ hybridization; RPKM, reads per kilobase million; TPM, transcripts per million.

killing against HCT116 cells that express endogenous MAGE-A3 (Fig. 5; Supplementary Fig. 4, Supplemental Digital Content 1, http://links.lww.com/JIT/A595). However, the MAGE-A3$_{112–120}$ CAR showed some nonspecific killing on HLA-A*02^{-} HCT116 cells at 3:1 E:T ratios. We believe this resulted from tonic signaling of the CAR (Supplementary

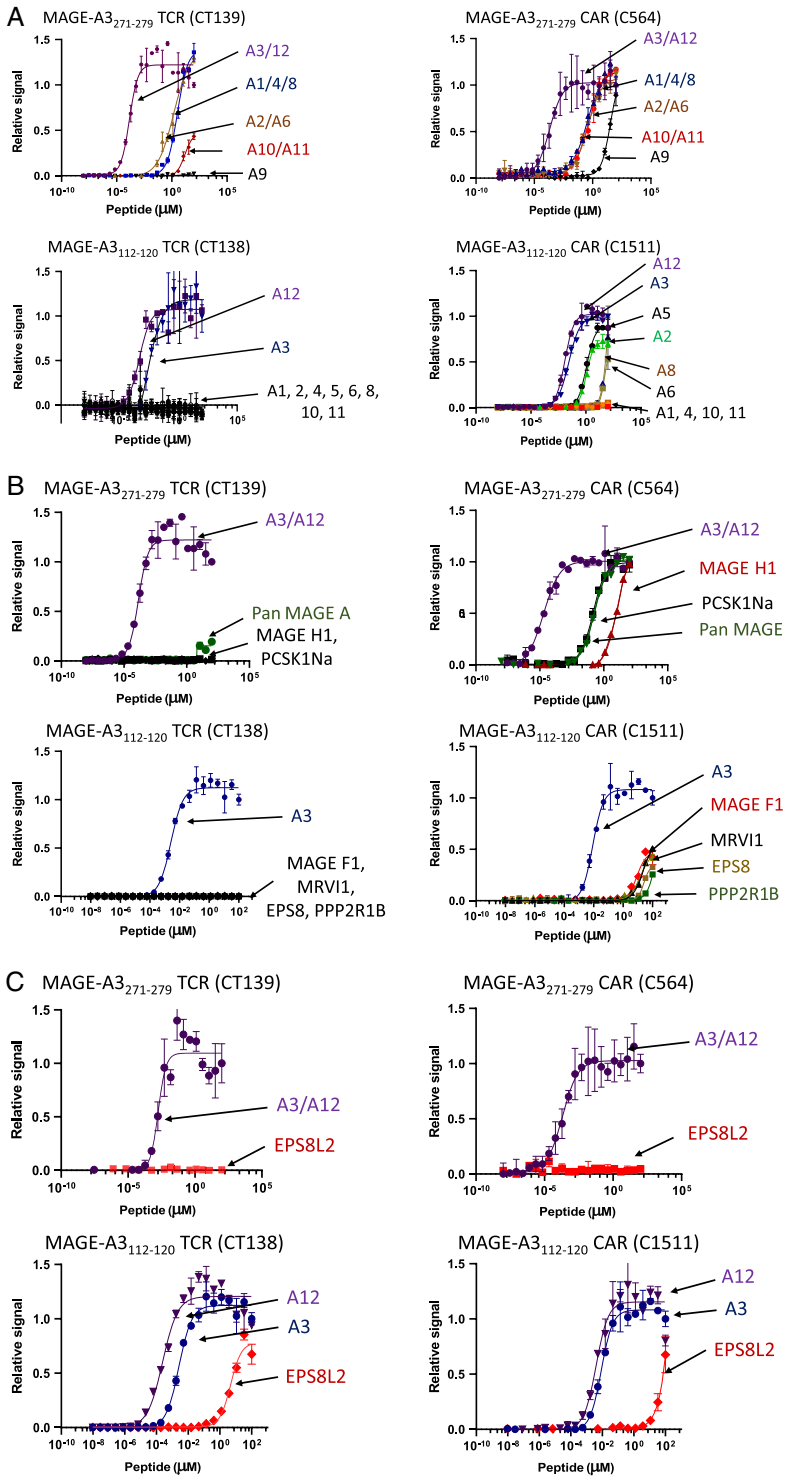


FIGURE 2. MAGE-A3 CAR and TCR selectivity in Jurkat/T2 cell assays. T2 cells were loaded with either on-target MAGE-A3 peptides or closely related peptides. Peptide-loaded T2 cells were cocultured with Jurkat cells expressing MAGE-A3 CARs or TCRs to assess their sensitivity against these peptides. In some cases, the same peptide sequence is shared among different MAGE-A family members. Each curve represents a different peptide sequence with its origin shown by text in corresponding colors: (A) selectivity against related peptides from MAGE-A family members; (B) selectivity against related peptides that are not from MAGE-A family members; (C) selectivity against EPS8L2. CAR indicates chimeric antigen receptors; TCR, T-cell receptor.

TABLE 3. Summary of Selectivity for MAGE-A3 CARs and TCRs Using Jurkat/T2 Assay and Primary T-cell Assays

		MAGE-A3$_{112\text{-}120}$ TCR (CT138)				MAGE-A3$_{112\text{-}120}$ CAR (C1511)			
Peptide name	**Peptide seq**	**MAGE-A3$_{112\text{-}120}$ EC50 (uM)**	**Selectivity window (EC50 ratio)**	**MAGE-A3$_{112\text{-}120}$ ECmin (uM)**	**Selectivity window (ECmin ratio)**	**MAGE-A3$_{112\text{-}120}$ EC50 (uM)**	**Selectivity window (EC50 ratio)**	**MAGE-A3$_{112\text{-}120}$ ECmin (uM)**	**Selectivity window (ECmin ratio)**
MAGE-A1	KVADLVGFL	>100	>10000	N/D	N/D	100	>2000	N/D	N/D
MAGE-A2	KMVELVHFL	53.4	5340	N/D	N/D	1.5	29	N/D	N/D
MAGE-A3$_{112\text{-}120}$ (on target)	KVAELVHFL	0.01	N/A	0.0003	N/A	0.05	N/A	0.0004	N/A
MAGE-A4	KVDELAHFL	>100	>10000	N/D	N/D	27	532	N/D	N/D
MAGE-A5	KVADLIHFL	>100	>10000	N/D	N/D	1.50	30	N/D	N/D
MAGE-A6	KVAKLVHFL	>100	>10000	N/D	N/D	33.8	676	N/D	N/D
MAGE-A8	KVAELVRFL	>100	>10000	N/D	N/D	33.5	670	N/D	N/D
MAGE-A10	KVTDLVQFL	>100	>10000	N/D	N/D	>100	>2000	N/D	N/D
MAGE-A11	KIIDLVHLL	>100	>10000	N/D	N/D	60.7	1214	N/D	N/D
MAGE-A12	KMAELVHFL	0.001	0.1	0.00002	0.067	0.007	0.14	0.0006	1.5
EPS8L2$_{339\text{-}347}$	SAAELVHFL	2.47	250	0.14	467	>100	>2000	1.23	3075
EPS8	SAADLVHFL	>100	>10000	N/D	N/D	25.3	506	N/D	N/D
MAGE-C3	KVAELVQFL	>100	>10000	N/D	N/D	24.4	488	N/D	N/D
MAGE-F1	TVAELVQFV	>100	>10000	N/D	N/D	8.50	170	N/D	N/D
MAGE-B18	KVVSLVHFL	>100	>10000	N/D	N/D	N/D	N/D	N/D	N/D
MRVI1	KLEELVHFL	>100	>10000	N/D	N/D	15.2	304	N/D	N/D
DDX28	KVAELVHIL	13.2	1320	N/D	N/D	N/D	N/D	N/D	N/D
		MAGE-A3$_{271\text{-}279}$ TCR (CT139)				MAGE-A3$_{271\text{-}279}$ CAR (C564)			
Peptide name	**Peptide seq**	**MAGE-A3$_{271\text{-}279}$ EC50 (uM)**	**Selectivity window**			**MAGE-A3$_{271\text{-}279}$ EC50 (uM)**	**Selectivity window**		
MAGE-A3$_{271\text{-}279}$ (on target)	FLWGPRALV	0.001	N/A			0.0007	N/A		
A1/A4/A8	FLWGPRALA	3.22	3200			0.35	500		
A2/A6	FLWGPRALI	1.43	1400			0.39	557		
A9	FLWGSKAHA	>100	>100000			20.3	29000		
A10/11	FLWGPRAHA	15.8	15800			0.53	757		
Pan-MAGE A	FLWGPRAL	8.20	8200			0.18	257		
MAGE-H1	FFWGPRAHV	>100	>100000			11.5	16428		
EPS8L2	SAAELVHFL	>100	>100000			>100	>142857		
MAGE-E2 pep1	FLWGSRAHRE	>100	>100000			>100	>142857		
MAGE-E2 pep2	FLWGSRAHR	>100	>100000			>100	>142857		
PCSK1Na	LLWGPRAGGV	>100	>100000			0.14	200		
PCSK1Nb	LLWGPRAGG	>100	>100000			32.0	45714		
IMMP2L-pep1	FLWFIVVLG	>100	>100000			>100	>142857		
IMMP2L-pep2	FLWFIVVL	>100	>100000			>100	>142857		
IgG VH	FAYWGPRAL	>100	>100000			12.4	17714		

Primary T cell cytotoxicity					
Peptide name	**Peptide seq**	**Cell lines**	**Assay**	**EC50 (uM)**	
				MAGE-A3$_{112\text{-}120}$ CAR (C1511)	**MAGE-A3$_{112\text{-}120}$ TCR (CT138)**
MAGE-A3$_{112\text{-}120}$	KVAELVHFL	MCF-7	Homogeneous	1.1	0.0007
		HEK293	QuIK	30	1.6
EPS8L2$_{339\text{-}347}$	SAAELVHFL	MCF-7	Homogenous	>100	0.005
		HEK293	QuIK	>100	1.4
MAGE-A12	KMAELVHFL	MCF-7	Homogeneous	4.0	0.0003
		HEK293	QuIK	36	0.27

The selectivity window is the ratio of EC50s or EC_{min} of on-target peptide over off-target peptide (eg, MAGE-A12:A3 or EPS8L2:A3). EC_{min} is the minimum peptide concentration with statistically significant difference from baseline.

(A) MAGE-A3$_{112–120}$ TCR and CAR in Jurkat/T2 assays; (B) MAGE-A3$_{271–279}$ CAR and TCR selectivity in T2/Jurkat cell assays; (C) Selectivity of MAGE-A3$_{112–120}$ CAR and TCR in primary T-cell assays with MCF7 or HEK293 cells loaded with peptide. For readout/assay details, see the Materials and methods section.

CAR indicates chimeric antigen receptors; NA, not applicable; ND, not determined; TCR, T-cell receptor.

Figs. 4C, D, Supplemental Digital Content 1, http://links.lww.com/JIT/A595).

Together, these data demonstrate that it is possible to obtain CARs with a selectivity profile similar to TCRs—discriminating among pMHCs that differ at only a single amino acid. Though less potent than the clinical TCR, the MAGE-$A3_{112–120}$ CAR was significantly more selective against $EPS8L2_{339–347}$, a homologous peptide that represents a risk for therapeutics directed against MAGE-$A3_{112–120}$. Because MAGE-A3 pMHCs, including HLA-A*02/MAGE-$A3_{112–120}$, are such attractive cancer targets, these results suggest it is worth reconsidering selective scFvs (and additional TCRs) as options for a MAGE-A3-directed therapeutic.

DISCUSSION

Cancer testis antigens are a class of potentially important tumor-selective proteins, so far not targeted by approved cancer medicine. They are remarkably cancer-specific, expressed in the testis, largely absent from other adult tissues, and overexpressed in a variety of tumor types.[23] The MAGE family fits this profile.[24] Of its paralogs, *MAGE-A3* is among the most interesting. It is expressed in small and squamous cell lung, head and neck, bladder, and esophageal carcinomas, and melanoma. In the subset of these cancer types that represents the upper quartile of MAGE-A3 mRNA expression, the levels range from 14 to 179 TPM (see TCGA database). This subset encompasses ~25,000 deaths/yr from cancer in the US. Thus, MAGE-A3 is an attractive cancer target based on its selective expression in tumors and its frequency and level of expression. Only a handful of proteins have these properties.

MAGE proteins reside inside the cell where they seem to function as modifiers of E3 ligase activity.[24] At present, the only known means of targeting them in cancer is through their display as pMHCs. The key challenges are to identify MAGE-A3 pMHCs on the cell surface and develop potent, selective targeting molecules. The A118T TCR was, therefore, an innovative, high-potential therapeutic when it began clinical testing.[2] However, the occurrence of fatal toxicity compelled the termination of this otherwise attractive candidate. The clinical findings raised the possibility of off-target TCR activity that Morgan and colleagues investigated. They proposed a reasonable hypothesis for the source of cross-reactivity, MAGE-A12, and provided supporting evidence. Given (i) the high similarity of *MAGE-A3* peptide to MAGE-A12 sequence, and the substantial challenge to avoid cross-reactivity with it; and, (ii) the potential high value of MAGE-A3 pMHC as a cancer target, it is sensible to re-examine the strength of the hypothesis.

Although it is impossible to exclude *MAGE-A12* completely as the root cause, the evidence presented here calls into question the hypothesis and provides evidence in favor of an alternative cause of toxicity: *EPS8L2*. Mindful of the inherent shortcomings of IHC, we utilized methodologies for mRNA expression that are comparatively more sensitive, quantitative, and less dependent on idiosyncratic reagents like antibodies. Notably, no positive or negative controls for antibody staining were included in the investigators' IHC experiments so the 6C1 antibody may be viewed as a poorly qualified reagent, especially for specific detection of low-level MAGE expression in situ. Moreover, the pattern of rare (1/100), strongly stain-positive cells may be viewed as highly unusual, especially for a protein with no known neural lineage–specific role. Given the totality of the results presented here and by Morgan et al, it seems more likely that the rare stain-positive cells imaged previously are false positives caused by, for example, spurious aggregation of dye. Our inability to detect the rare, strongly $MAGE^+$ cells in the brain predicted by the hypothesis at the RNA level raises questions about the actual source of toxicity observed in the TCR-T trial. Nonetheless, it remains possible that despite a thorough search we missed rare MAGE-A12-expressing cells responsible for initiating the neurotoxicity in the brain sections we examined. In addition, despite certain advantages of RNA staining compared with IHC, RNA FISH has its own limitations for sensitivity, including the inherent instability of RNA and, despite positive and negative controls, it is conceivable that MAGE-A3 has some unusual properties that interfere with its detection through RNA FISH.

The clinical TCR did not react with a variety of cell lines and tissues, at least some of which likely expressed *EPS8L2*.[2] However, in our hands, the TCR clearly mediated a response using an *EPS2L8*-expressing cell line as a stimulus. These disparate results may reflect sensitivity differences in the assays used. Based on (i) the ~100× higher expression of EPS8L2 compared with MAGE-A12 in normal tissues, including brain (Supplementary Figure 1, Supplemental Digital Content 1, http://links.lww.com/JIT/A595); (ii) the detection of $EPS8L2_{339–347}$ peptide by mass spectrometry among pMHC complexes immunoprecipitated from a cell line; and, (iii) the significant response of the TCR to both $EPS8L2_{339–347}$ peptide-loaded cell lines and cells expressing full-length EPS8L2 protein, we believe $EPS8L2_{339–347}$ should be considered as a likely cause of neurotoxicity observed in the clinical study of the A118T TCR.

Although we believe ESP8L2 is a strong candidate, there are other related peptides that cannot be completely excluded. However, we tested a set of peptides from the proteome most closely related to MAGE-$A3_{112–120}$, and none reacted with the TCR as strongly as MAGE-$A12_{112–120}$ and $EPS8L2_{339–347}$ (Figs. 2A–C; Table 3A). In addition, it is well known that TCRs can cross-react with peptides that share limited sequence similarity. One well-known example involves another MAGE-A3 affinity-matured TCR-T directed at a different MAGE-A3 peptide in complex with HLA-A*01. After 2 patient deaths on trial, the investigators traced the problem to potential cross-reaction with a peptide derived from the cardiomyocyte-specific protein, titin.[1,25,26] The titin peptide shares only 5 of 9 residues with the on-target MAGE peptide. Given results like these, *ESP8L2* should be viewed as a likely but still unproven source of the clinical neurotoxicity of the A118T TCR. It remains possible that this TCR, because of its unique binding surface features, reacts with an off-target molecule in the body that has so far eluded detection.

To provide support for a potential alternative route to a MAGE-A3 therapeutic, we isolated CARs using 2 different HLA-A*02-restricted MAGE-A3 peptides. Because it is impossible to rule out MAGE-A12 entirely as a safety risk, we attempted to minimize cross-reactivity of the MAGE-$A3_{112–120}$ CAR by counter-screening against MAGE-A12. We managed to improve selectivity compared with the TCR by ~10×. Optimization against ESP8L2 was also successful; the MAGE-$A3_{112–120}$ CAR demonstrated ~10× improvement in selectivity against $EPS8L2_{339–347}$ compared with the TCR. The 2 MAGE-A3-directed CARs, like the TCR, were otherwise selective, reacting minimally against a panel of related and unrelated peptides, including other members of the MAGE family.

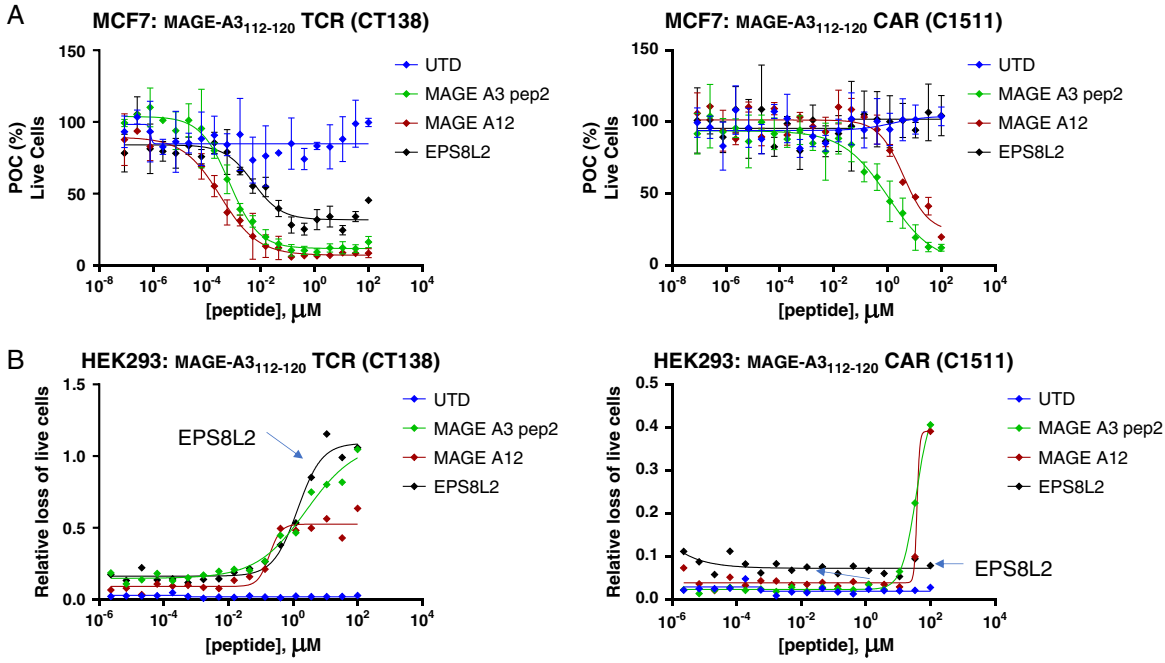


FIGURE 3. Primary T-cell cytotoxicity of MAGE CARs and TCRs on peptide-loaded MCF7 and HEK293 (see Table 3C for the summary). Different assay formats for the cell lines were required because we were unable to create an effective HEK293 luciferase-reporter line. A, Homogeneous cytotoxicity assays were used on MCF7 cells (no/low expression of MAGE-A; positive expression for EPS8L2). B, The selectivity against EPS8L2 was confirmed in HEK293 cells (no/low expression of MAGE and EPS8L2) using the imaging-based QuIK assay. Averages of 2 measurements are plotted. CAR indicates chimeric antigen receptors; POC, percentage of control; TCR, T-cell receptor; UTD, untransduced T cells.

Given the unusual, highly desirable, expression attributes of *MAGE-A3* in the oncology context, CARs with these selectivity features may be options for T-cell therapy. The emergence of improved methods to screen for mAbs that interact selectively with closely related pMHC targets suggests that it may be possible to exploit antibody-based ligand-binding domains, in addition to clinical TCRs, as a source of therapeutic entities for MAGE-A3 pMHCs. We were able to obtain an scFv with reduced cross-reactivity risks of both potential off-target peptides. We believe that either a CAR or TCR with such improved selectivity properties and TCR/CARs directed at the other defined MAGE-A3/12 pMHC target represent options worth considering in light of the data described here. Recognizing the risk that accompanies potential benefit, additional safety measures for MAGE-A3-directed therapeutics may also be advisable. These include close monitoring of dosed patients, rapid application of anti-inflammatory treatments in the event of adverse responses [eg, glucocorticoids, interleukin (IL)-6 antibody], and/or a safety switch engineered into the construct.[27]

MAGE-A3 vaccines have so far shown unspectacular results in the clinic. A recombinant-protein-based vaccine failed to demonstrate efficacy in a phase 3 trial of lung cancer patients.[28] These disappointing results may be attributed to

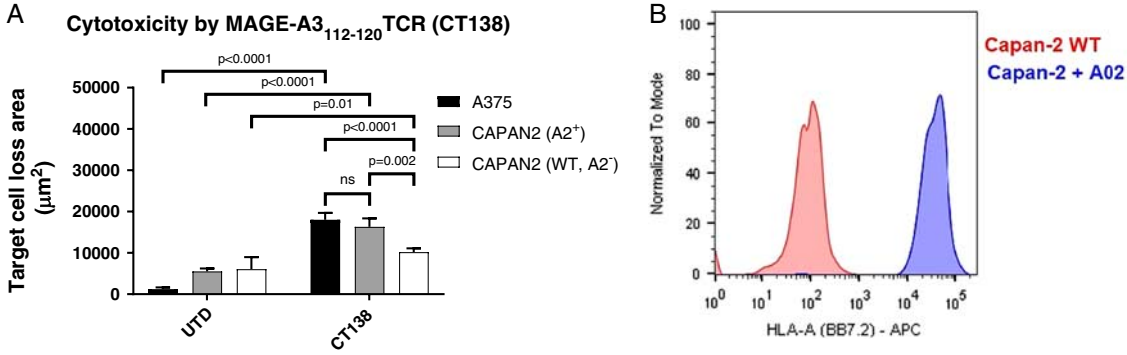


FIGURE 4. Cytotoxicity assay for the MAGE-A3$_{112–120}$ TCR in A375 and CAPAN-2 cell lines. MAGE-A3$_{112–120}$ TCR showed similar level of cytotoxicity on A375 (MAGE-A3+) and CAPAN-2 (MAGE-A3-, EPS8L2+) cell lines when the HLA-A*02 is recombinantly overexpressed, indicating this TCR can interact with a target other than MAGE-A3, likely EPS8L2. In wild-type CAPAN-2 (HLA-A*02^{-}), the cytotoxicity level was significantly reduced. A, Cytotoxicity. B, Cell surface staining of HLA on CAPAN-2 cells ± HLA-A*02 expression. TCR indicates T-cell receptor; UTD, untransduced T cells; WT, wild type.

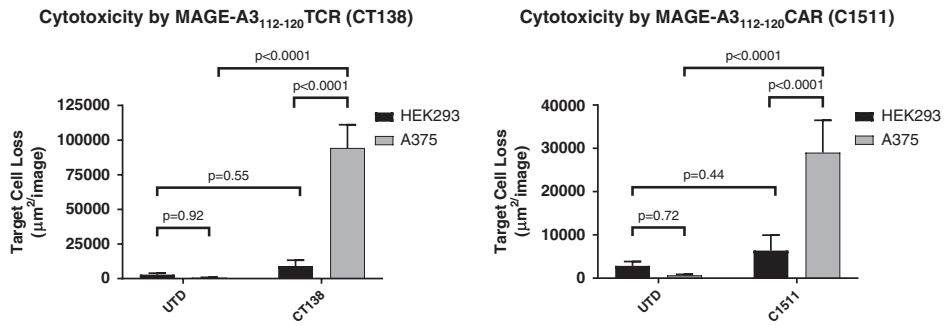


FIGURE 5. Cytotoxicity of MAGE-A3 CAR on cell lines that endogenously express MAGE-A3. Selective killing of A375 (MAGE+) and HEK293 (MAGE^{-}/EPS8L2^{-}; negative control) cells by MAGE-A3 TCR (CT138, left) or MAGE-A3 CAR (C1511, right). CAR indicates chimeric antigen receptors; TCR, T-cell receptor.

problems with MAGE-A3 as an immuno-oncology target. However, we believe that the key difference between vaccines and TCR/CAR-Ts is that the latter does not require an immune response. The effectors are engineered with the desired activity. Therefore, given the selective expression of MAGE-A3 and proven display of endogenous MAGE-A3 pMHCs, in our view, MAGE-A3 deserves further consideration as a cell therapy cancer target.

MATERIALS AND METHODS

Cell Lines and Peptides

A375 cells (CRL-1619), CaSKI (CRM-CRL-1550), PANC.1 (CRL-1469), SW527(CRL-7940), PC.3 (CRL-1435), LNCaP_clone_FGC (CRL-1740), MCF.7 (HTB-22), CAPAN-2 (HTB-80), HEK293T (CRL-11268), T2 (CTL-1992), and HCT116 (CCL-247) were purchased from ATCC. VMRC.LCD (JCRB0814) was purchased from JCRB Cell Bank. Jurkat NFAT-Firefly-Luciferase cells were purchased from BPS Bioscience (#60621). All cell lines were cultured in media as recommended by the vendors. A 100 U/mL penicillin-streptomycin (Gibco 15140163) (1× P/S) was used in all media. Renilla luciferase was recombinantly expressed in MCF7 cells, and these recombinant cells were cultured in their respective culture media supplemented with 400 µg/mL Geneticin. The *HLA-A*02* gene was recombinantly expressed in CAPAN-2 cells. HLA-A*02 was knocked out in HCT116 using CRISPR technology as described previously.[29] Suspension cells were maintained below a density of 1E6/mL. Adherent cell lines were passaged at ~80% confluency.

All peptides were purchased from GenScript by custom order.

HuTARG Sort

HuTARG primary libraries were from Innovative Targeting Solutions, Inc. An in vitro V(D)J repertoire with >1 billion diversity was generated by expression of *RAG-1* and *TdT* in the host cells as described previously.[21,22] pMHC probes were generated as described previously.[30] The library was enriched for cells displaying antibodies that bind specifically to target pMHC probes, but not to off-target pMHC probes using a flow sorter device. Multiple enrichment rounds were performed to increase on-target and decrease off-target binding. In the final round, on-target and off-target binding cells were collected. RNA was extracted from these pools and reverse transcribed into cDNA. polymerase chain reaction fragments containing the complementarity-determining regions (CDR) regions were generated using the cDNAs as a template, followed by targeted next-generation sequencing (NGS) to determine the frequency of each binder with a unique CDR region. The degree of enrichment/depletion was determined by comparing the output and input NGS counts.

Target-specific binders from the primary libraries were used in some cases as parents to generate optimization libraries to further improve on-target sensitivity and/or reduce off-target cross-reactivity. Optimization libraries were constructed by diversification of CDR-1, CDR-2, or CDR-3 light chains of parent binders by in vitro RAG-mediated V(D)J recombination. The optimization library was enriched for on-target activity and depleted for off-target activity as for enrichment of the primary library. NGS was also used to identify binders enriched as previously described.

Molecular Cloning

All CAR constructs were created by fusing an scFv ligand binding domain to a hinge, a transmembrane, and an intracellular signaling domain. The hinge was derived from CD8, the transmembrane domain from CD28, and the signaling domain from CD28, 4-1BB, and CD3. Gene segments were combined using Golden Gate cloning and inserted downstream of a human Elongation factor 1-alpha promoter contained in a lentivirus expression plasmid.

Jurkat/T2 Cell Assay

Jurkat NFAT-Firefly-Luciferase cells were transfected on day 1 with TCR and CAR constructs using standard protocols for the Lonza 4D Nucleofector (AAF-1002B). T2 cells were loaded with peptides listed in Tables 1 and 3. Peptides were resuspended in dimethyl sulfoxide, and diluted 16 or 20 times serially 3× per step. Serially diluted peptide solutions were added to T2 cells resuspended in peptide-loading media (Roswell Park Memorial Institute (RPMI) 1640 (medium) + 1% Bovine serum albumin + 1× P/S). This yielded peptide-loaded T2 cells at ~1E6/mL, with peptide concentrations ranging from ~10 fM to 100 µM, including a control at 0 µM. Peptide-loaded T2 cells were incubated overnight at 37°C in 384-well plates (Thermo Scientific AB0781). On day 2, the cells were cocultured in a 384-well plate (Corning 3570). Peptide-loaded T2 cells (10,000 cells/well) were added to CAR/TCR-transfected Jurkat-NFAT-Firefly-Luciferase cells (12,000 cells/well) to a final volume of 20 µL. After a 6-hour incubation at 37°C, the One-Step" Luciferase assay system (Firefly luciferase, BPS Bioscience, 60690) was used to determine luminescence intensity on a Tecan Infinite M1000.

Primary T-Cell Transduction

Human peripheral blood mononuclear cells were purified from Leukopaks purchased from Allcells according to the

method described by Garcia et al[31] Collection protocols and donor informed-consent were approved by an institutional review board at Allcells. Allcells followed Health Insurance Portability and Accountability Act compliance and approved protocols (https://www.allcells.com/cell-tissue-procurement/donor-facilities/). Unless otherwise specified, all LymphoONE media (Takara WK552) was supplemented with 1% human AB Serum (GeminiBio 100-512). Human peripheral blood mononuclear cells were grown in LymphoONE and supplemented with TransAct (Miltenyi 130-111-160) following the manufacturer's guidelines (1:100 dilution) for 24 hours before being transduced with lentivirus encoding a CAR or TCR. Twenty-four hours after transduction, additional LymphoONE supplemented with IL-2 (300 IU/mL) was added to transduced cells and cultured for 3 days before transfer to a 24-well G-Rex plate (Wilson Wolf 80192M). Fresh IL-2 (300 IU/mL) was added every 48 hours with a media change every 7 days during expansion in G-Rex plates. Expression and antigen binding of transduced CARs or TCRs in primary T cells were confirmed by flow cytometry as previously described. If needed, CAR- or TCR-expressing cells were labeled with protein L-biotin/streptavidin-PE or mTCR-PE, followed by anti-PE microbeads (Miltenyi 130-048-801) according to the manufacturer's protocol, and subsequently enriched using AutoMACS Pro Separator (Miltenyi). Enriched cells were grown in G-Rex plates until harvest.

Homogeneous Cytotoxicity Assay

Target cells (MCF7 Renilla luciferase) were loaded with target peptides as previously described in the Jurkat/T2 section, except that LymphoONE supplemented with 1% human serum and 1× P/S was used. Twenty-four hours after peptide loading, a calibration curve was generated using CellTiter-Glo (Promega G7570) readout to determine the number of target cells seeded per well. T cells were mixed with target cells at 3:1 E:T ratio according to the target cell number determined by calibration. After 48-hour coculture at 37°C, cytotoxicity of primary T cells was quantified by the bioluminescence using the Renilla Luciferase Assay System (Promega, E2810) on Tecan Infinite M1000.

QuIK Assay

QuIK assay method was the same as described elsewhere.[32] Adherent target cells (MCF7) were cultured under standard conditions and seeded at 2000 cells per well in LymphoONE media containing 1% human serum in 384-well plates (Greiner 781091). Serially diluted peptide solutions were added to the target cells to yield peptide concentrations ranging from ~2 pM to 100 μM. Peptide-loaded target cells were incubated at 37°C overnight. Fresh 5-chloromethylfluorescein diacetate (Invitrogen C7025) working solution at 1 mM was prepared in LymphoONE from the solid. The percentages of TCR^- and CAR^+ T cells, determined by flow cytometry, were used to adjust T-cell numbers for coculture with target cells. T cells were harvested and resuspended in 5-chloromethylfluorescein diacetate working solution at 200,000 cells/mL, and incubated in the dark at 37°C for 30 minutes. After staining, the cells were centrifuged and washed 2× with 1 mL LymphoONE media containing 1% human serum. The cells were counted and added at an E:T ratio of 1:1 to the peptide-loaded target cells. Annexin V Red (Sartorius 4641) was dissolved according to the manufacturer's instructions, and added to the coculture at a final dilution of 1:200; propidium at a final dilution of 1:10,000 (ThermoFisher P3566). Immediately after adding T cells to the peptide-loaded target cells, the plate was incubated in an IncuCyteTM S3 and scanned every 2 hours using phase, green, and red channels with 300 ms exposure for the fluorescent channels at ×10 magnification.

Cytotoxicity Imaging Assay ("Inside Out" Presentation/Killing)

To compare the killing of target cells expressing MAGE (on-target) versus ESP8L2 (off-target), 2 target cell lines were selected for a cytotoxicity study (without any peptide loading): $A*02:01^+$ A375 (*MAGE-A3*$^+$/*MAGE-A12*$^+$/*EPS8L2*^{low}) cells and $A*02:01^-$ CAPAN-2 ($MAGE\text{-}A3^-$/$MAGE\text{-}A12^-$/$EPS8L2^+$). To provide control to demonstrate HLA restriction of TCR specificity, *HLA-A*02* gene was recombinantly expressed in wild-type (WT) CAPAN-2 cells. In addition, HCT116 ($MAGE\text{-}A3^+$/$EPS8L2^+$) cells were used to demonstrate the cytotoxicity effect is HLA-A*02-restricted. CRISPR/Cas9 editing was used to knock-out the *HLA-A*02:01* locus in HCT116, creating an HLA- $A*02^-$ version of this cell line. Adherent target cells (A375, CAPAN-2 WT, CAPAN-2 $A*02^+$, HCT116 WT, or HCT116 $A*02^-$) were cultured in standard conditions and seeded at 2000 cells per well in LymphoOne media containing 1% human serum in 384-well plates (*Greiner 781091*). Two thousand target cells were incubated at 37°C overnight. The next day, untransduced and TCR^+ T cells were counted and added to the plated target cells at indicated E:T ratios. In addition, Annexin V Red (Sartorius 4641) was resuspended according to the manufacturer's instructions and added to the coculture at a final dilution of 1:200. Propidium iodide was added at a final dilution of 1:10,000 (ThermoFisher P3566). Immediately after adding T cells to seeded target cells, the plate was placed in the IncuCyteTM S3 and scanned every 2 hours using phase, and red channels with 300 ms exposure for the fluorescent channels at ×10 magnification.

RNA FISH (BaseScope)

The BaseScope experiments were performed by Advanced Cell Diagnostics (Newark, CA; a Biotechne brand) under a service contract agreement. The probe design used 1 pair of ZZ probes at nucleotide location: 91–127 nt of MAGE-A12 (NM_005367.6, Fig. 1A). Two human Formalin-Fixed Paraffin-Embedded cell pellet samples (PC3, and K562) and 8 human Formalin-Fixed Paraffin-Embedded brain samples were evaluated by BaseScope LS Red ISH. Human samples, Peptidyl-prolyl cis-trans isomerase B was used as a positive-control marker for sample QC and to evaluate RNA quality in both cell pellets and tissue samples. The bacterial gene dapB was used as a negative control. Optimization was performed to establish the best signal-to-noise ratio. Optimization of this probe set yielded mild pretreatment assay conditions (Epitope Retrieval 2: 15 min at 88°C; Protease III: 15 min at 40°C) for the cell pellets and standard pretreatment assay conditions (Epitope Retrieval 2: 15 minutes at 95°C; Protease III: 15 min at 40°C) for tissue samples. All samples passed QC with moderate Peptidyl-prolyl cis-trans isomerase B^+ control staining and little to no dapB background staining. This established BaseScope LS Red ISH assay was performed to evaluate 2 cell pellets and in human brain samples. THE specific RNA staining signal was identified as red, punctate dots. Samples were counterstained with Mayer's Hematoxylin.

pMHC Peptide Identification From Cell Lines by Mass Spectrometry

Mass spectrometry experiments were conducted by Caprion Biosciences (Montreal, Canada) by contact. PANC.1, A.375, SW527, CaSki, PC.3, LNCaP_clone_FGC, MCF.7, and VMRC.LCD were cultured as previously described. They

were dissociated using cell dissociation buffer (ThermoFisher 13151014). pMHC complexes were purified from ~300 to 500 million cells of each cell line by immunoaffinity chromatography using the anti-MHC-I antibody W6/32 from cell lysates. pMHC complexes were disrupted with a mild acid. The presented peptides were desalted with a mixed cation exchange matrix and analyzed by LC-MS/MS in a single injection using nanoflow reverse-phase liquid chromatography (NanoAcquity UPLC, Waters) coupled to a high-resolution mass spectrometer (Q Exactive, Thermo Scientific). Mass spectrometry data were analyzed using Elucidator software (Rosetta), Mascot software (Matrix Science), and PEAKS software (Bioinformatics Solutions Inc.) to determine peptide sequences and relative quantity in each sample. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium through the PRIDE partner repository with the data set identifier PXD022020.

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Conflicts of Interest/Financial Disclosures

All authors are employees of A2 Biotherapeutics Inc.

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