Supplementary Online Content

Ahmed N, Brawley V, Hegde M, et al. HER2-specific chimeric antigen receptor–modified virus-specific T cells for progressive glioblastoma: a phase 1 dose-escalation trial. *JAMA Oncol.* Published online April 20, 2017. doi:10.1001/jamaoncol.2017.0184

This supplementary material has been provided by the authors to give readers additional information about their work.

eAppendix. Materials and Methods

Generation of HER2-CAR VSTs

Autologous HER2-CAR modified virus-specific T-cells (HER2-CAR VSTs) were manufactured from peripheral blood mononuclear cells (PBMCs) obtained from a blood draw according to current Good Manufacturing Practice (cGMP) guidelines as previously described.[1,2] PBMCs were transduced with a clinical grade adenoviral vector encoding the immunodominant CMV pp65 antigen (Ad5f35pp65) after an overnight adherence step.[1,2] Starting on day 10 post transduction, the cells were re-stimulated weekly with irradiated autologous EBV-transformed lymphoblastoid cell lines (LCLs) transduced with the same Ad5f35pp65 vector (Ad5f35pp65-LCL). Three to 4 days after the 2nd Ad5f35pp65-LCL stimulation T-cells were transduced with a clinical grade retroviral vector encoding a HER2-specific CAR, consisting of a murine scFv FRP5, a short hinge, a CD28 transmembrane domain, and a CD28.ζ signaling domain.[3,4] HER2-CAR VSTs were cryopreserved 7 to 10 days after the 4th stimulation.

Flow cytometry

A FACSCalibur instrument (Becton Dickinson, San Jose, CA) and CellQuest software (Becton Dickinson) was used for flow cytometric analysis.[4] Monoclonal antibodies (MAbs) were obtained from Becton Dickinson and included anti-CD3, -CD4, -CD8, -CD16, -CD19, -CD56, -CD62L, -CCR7, -TCRαß, and -TCR $\gamma\ \delta$. HER2-CAR expression was detected with a murine scFV-specific MAb (Jackson ImmunoResearch Laboratories). Negative controls included isotype antibodies.

Real-time PCR assay

We used a FRP5-specific primer and TaqMan probe (Applied Biosystems) to detect HER2-CAR T cells.[4] DNA was extracted with the QIAamp DNA Blood Mini Kit (Qiagen) and qPCR was performed in triplicates using the ABI RPISM 7900HT Sequence Detection System (Applied Biosystems). The baseline range was set at cycles 6-15, with the threshold at 10 SDs above the baseline fluorescence. To generate DNA standards, we established serial dilution of DNA plasmids encoding each specific cassette.

Enzyme-linked immunospot (Elispot) assay

The frequency of antigen-specific T cells in the HER2–CAR VST product and peripheral blood of patients was measured using interferon-γ (IFN-γ) Elispot assays as previously described.[1,5] Briefly, HER2-CAR VSTs or PBMCs were stimulated with overlapping peptide mixes for pp65, IE1, hexon, and penton. Peptide mixes contained 15 amino-acid peptides covering the entire length of the corresponding protein with an 11 amino-acid overlap (pepmixes; JPT Peptide Technologies, Berlin, Germany). Media (no peptide) served as negative control, and Phytohemagglutinin (PHA, Sigma) as positive control. Developed Elispots were analyzed by ZellNet Consulting (New York, NY). Spot-forming cells (SFCs) were calculated and expressed as SFC per 10^5 cells for T-cell products and $2x10^5$ cells for PBMCs.

Cytotoxicity assay

Cytotoxic activity of HER2-CAR VSTs against targets was determined by standard ^{51}Cr release assay.[3] $1x10^6$ target cells were labeled with 50μCi ^{51}Cr and incubated for 1 hour. Targets were then washed and $5x10^3$ cells were co-cultured with effector T cells at different effector to target (E:T) ratios. Supernatants were analyzed with a Packard Cobra Quantum gamma counter Model E 5010 (Perkin Elmer, Shelton CT) reader after 4 hour incubation. Lysis was calculated as previously described.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin embedded sections (6μm) of GBM were processed as previously described,[5-7] and stained with a phospho-HER2 MAb (CB11, Abcam, Cambridge, MA) for HER2 detection. All slides were counterstained in Harris hematoxylin. Known HER2-expressing breast cancer samples were used as positive controls. Slides only stained with secondary MAb served as negative controls.

eTable 1. Patient Characteristics

UPN	Age (years) /sex	Dx	Prior Treatment				
			Surgery	XRT+TMZ	Salvage Therapies	Investigational Agents	Time to T-Cell Therapy (months)
01	42.8/F	GBM	Yes, x3	Yes	(1) TMZ + Hydrox (2) TMZ (3) CCNU + Bev (4) TMZ + Hydrox + Bev (5) Irino + Bev	(1) TMZ + Iniparib (2) Carbo + Iniparib	27.2
02	59.3/M	GBM	Yes, at dx	Yes	Bev	None	12.4
03	29.6/M	GBM	Yes, at dx	Yes	(1) BCNU (2) Bev	Veliparib	16.0
04	17.1/M	GBM	Biopsy only	Yes	None	None	7.3
05	62.2/M	GBM	Yes, x2	Yes	Irino + Bev	None	27.2
06	59.4/F	GBM	Yes, x2	Yes	TMZ + Accutane + Verapamil + Metformin + Tamox	(1) Toca 511 + Toca (2) Bev + EGFRvIII vaccine	20.3
07	61.7/F	GBM	Yes, at dx	Yes	Bev + Carbo	Imatinib mesylate	13.3
08	10.6/M	GBM	Yes, at dx	XRT, No TMZ	None	None	7.3
09	63.5/M	GBM	Yes, x2 (STR then GTR)	Yes	None	Veliparib	12.8
10	50/M	GBM	Yes, at dx	Yes	Paclitaxel	None	13.2
11	62.7/M	GBM	Yes, at dx	Yes	None	None	11.3
12	13.1/M	GBM	Yes, x2	Yes	Bev	None	6.2
13	69.3/F	GBM	Yes, at dx	Yes	None	None	17.0

14	14.4/M	GBM	Yes; STR x2	XRT, no TMZ	None	None	16.7
15	14.4/F	GBM	Yes, x4	XRT, no TMZ	(1) Vori (2) Dasatinib	5-FU + IFNα2b	5.9
16	10/F	GBM	Yes, x2	Yes	None	None	9.2
17	16.2/F	GBM	Yes, at dx	Yes	TMZ + CCNU	None	12.9

GBM: Glioblastoma
Dx: Diagnosis
XRT: radiation therapy
TMZ: Temozolomide
UPN: unique patient number
CCNU: Lomustine
Bev: Bevacizumab
Irino: Irinotecan
Hydrox: Hydroxychloroquine
Tamox: Tamoxifen
Carbo: Carboplatin
Vori: Vorinostat
BCNU: Carmustine
IFN: Interferon
STR: subtotal resection
GTR: gross total resection
Toca 511: Vocimagene amiretrorepvec

eTable 2. Expression of HER2, CMV pp65, and CMv IE1 in GBMs of Study Patients

UPN	HER2		CMV			
			pp65		IE1	
	Intensity	Grade	Intensity	Grade	Intensity	Grade
1	2+	2	-	-	-	-
2	2+	1	0	0	1+	Grade 1
3	1+	1	NE	NE	NE	NE
4	2+	4	0	0	0	0
5	3+	2	0	0	0	0
6	2+	2	NE	NE	0	0
7	2+	2	1+	1	1+	1
8	1+ to 2+	1	1+	1	1+	1
9	3+	2	0	0	1+	1
10	2+	2	0	0	1+	1
11	3+	2	0	0	0	0
12	2+	1	0	0	0	0
13	3+	1	2	2	0	0
14	2+	2-3	0	0	0	0
15	2+	1	1+	4	1+	4
16	2+	3	0	0	0	0
17	3+	2	1+	3	2+	4

*Grade (cells positive): 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100%
**Intensity: 1+ to 3+ based on positivity of control slides
NE: not evaluable

eTable 3. Patient Outcomes

UPN	Site of tumor at T-cell infusion; measurement	T-cell dose/m^2	Disease Reponses (6 weeks)	Time to Progression (months from first infusion)	Survival (in months)		Outcome
					From first T-cell infusion	From diagnosis	
01	Genu of the corpus callosum and left forceps minor; irregular shape >1 cm thick	1×10^6 3×10^6 1×10^7 3×10^7	SD	4.4	27.8	55.0	DOD
02	Right parietal lobe; irregular shape >1 cm thick	1×10^6	PD	4.0	4.0	16.4	DOD
03	Left temporal lobe; 3 cm	1×10^6	PD	2.1	15.5	31.5	DOD
04	Right thalamic lesion; 4 x 3 cm	1×10^6 (x2)	PR	9.2	26.9	34.2	DOD
05	Left frontal lobe; 4.7 x 3.8 cm	3×10^6	PD	3.6	3.7	30.9	DOD
06	Left parietal lobe; 4.6 x 3.8 cm	3×10^6	SD	2.3	2.4	22.7	death from peritoneal bleed
07	Corpus callosum; 2 x 0.7cm	3×10^6	PD	1.4	6.9	20.3	DOD
08	Right fronto-parietal cortex; stellate >1 cm thick	1×10^7	SD	no progression	28.6	35.9	Alive
09	Temporo-parietal; 1 cm rim enhancement >1 cm thick	1×10^7 (x6)	SD	no progression	28.4	41.2	Alive
10	Right parietal, right pulvinar region, right periventricular, anterior insular cortex (multifocal) >1 cm	1×10^7	PD	0.8	10.9	24.1	DOD
11	Rim enhancement; 1 cm thick	3×10^7	SD	lost to follow up at 6 months	7.9	19.2	DOD
12	Rim enhancement; 1 cm thick	3×10^7	PD	1.1	2.7	8.9	DOD
13	Frontal lobe rim enhancement; 1 cm thick	3×10^7 (x 3)	SD	no progression	23.7	40.7	Alive
14	Left temporal lobe; 3.2 x 1.5 cm	3×10^7	PD	1.2	6.1	22.8	DOD
15	Bilateral frontal lobe butterfly lesion; 8.3 x 6.7 x 6.5 cm	1×10^8 (x2)	SD	2.7	6.4	12.3	DOD
16	Right temporal lobe lesion; 2.9x1.6cm	1×10^8 (x 2)	PD	1.3	7.8	17.0	DOD
17	Left thalamus; 2.2 x 1.2 cm lesion	1×10^8 (x 2)	PD	3.5	11.3	24.2	DOD

PD: progressive disease
SD: stable disease
PR: partial response
DOD: died of disease

eTable 4. Univariate Cox Proportional Hazards Regression Analysis

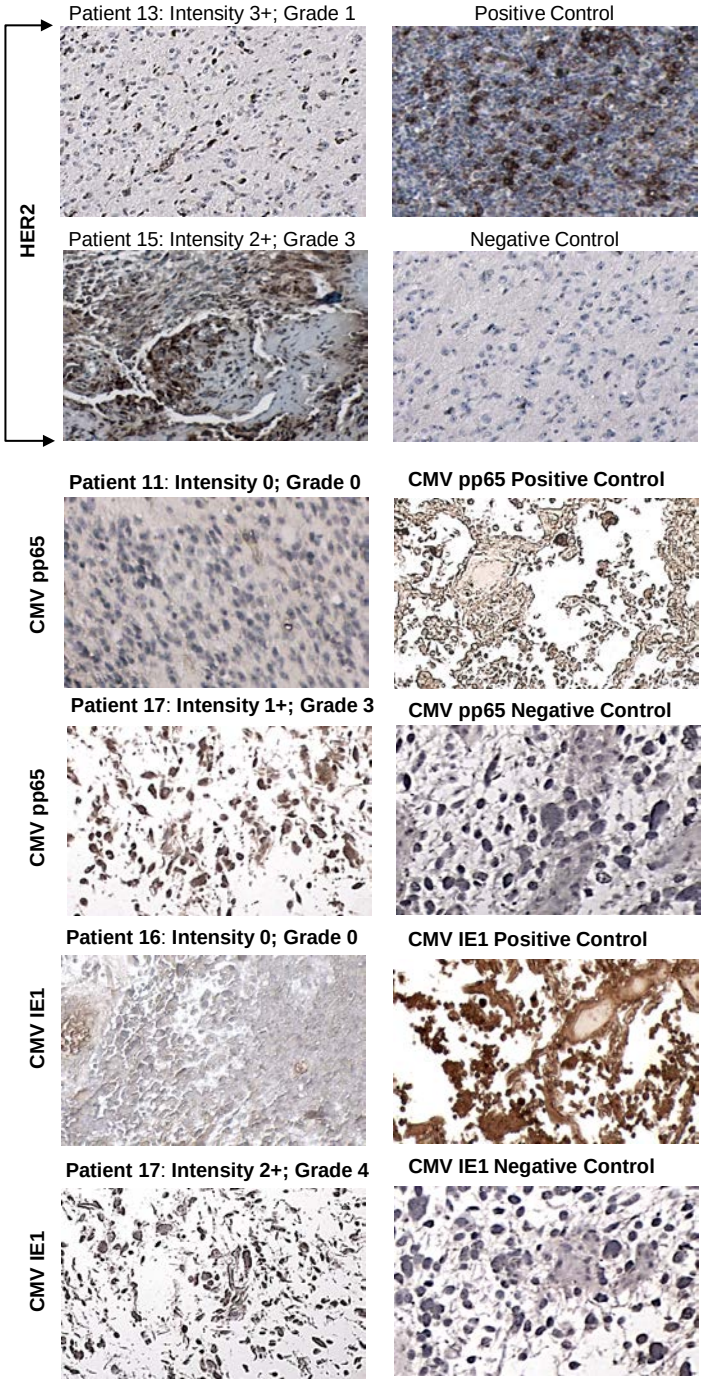
Variable	Progression free survival		Overall survival	
	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
Age at diagnosis				
≤18 years (n=7)	1.519 (0.505-4.567)	0.457	1.252 (0.419-3.743)	0.688
>18 years (n=10)	1		1	
Sex				
Female (n=7)	1.384 (0·457-4·189)	0.565	1.312 (0.437-3.941)	0.629
Male (n=10)	1		1	
Salvage therapy				
No (n=7)	1		1	
Yes (n=10)	6.24 (1.291-30.161)	0.023	4.302 (1.16-15.958)	0.018
Time to T-cell therapy from dx				
≤14 months (n=11)	1		1	
>14 months (n=6)	1.171 (0.381-3.6)	0.783	1.27 (0.41-3.932)	0.678
HER2 expression grade[a]				
<2 (n=6)	1		1	
≥2 (n=11)	1.389 (0.426-4.532)	0.586	1.203 (0.367-3.944)	0.761
HER2 expression intensity[b]				
3 (n=5)	1		1	
<3 (n=12)	4.203 (0.922-19.16)	0.064	2.788(0.615-12.635)	0.183
Number of T-cell infusions				
Single (n=10)	1.889 (0.605-5.894)	0.273	2.265 (0.723-7.092)	0.161
Multiple (n=7)	1		1	
T-cell dose level				
1 (n=4)	1		1	
2 (n=3)	2.231 (0.439-11.33)	0.333	8.415 (1.237-57.25)	0.030
3 (n=3)	0.307 (0.033-2.832)	0.298	0.237 (0.026-2.177)	0.203
4 (n=4)	0.665 (0.121-3.655)	0.639	1.486 0.233-9.481)	0.675
5 (n=3)	2.437 (0.465-12.776)	0.292	2.639 (0.461-15.111)	0.276
T-cell phenotype				
CD8+/CD4+ ratio (n=17)	0.981 (0.948-1.015)	0.264	0.982 (0.951-1.014)	0.265
CD3+/RO+ (n=17)	1.073 (0.945-1.218)	0.278	1.045 (0.919-1.189)	0.502
CD3+/RO+/	0.993 (0.95-1.038)	0.757	1.009 (0.962-1.058)	0.720

CD62L+ n=17)						
CD3+/RO+/CCR7-/CD62L- (n=17)	1.011 (0.969-1.055)		0.616	0.998 (0.954-1.043)		0.922

[a]Grade (cells positive): 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100%

[b]Intensity: 1+ to 3+ based on positivity of control slides

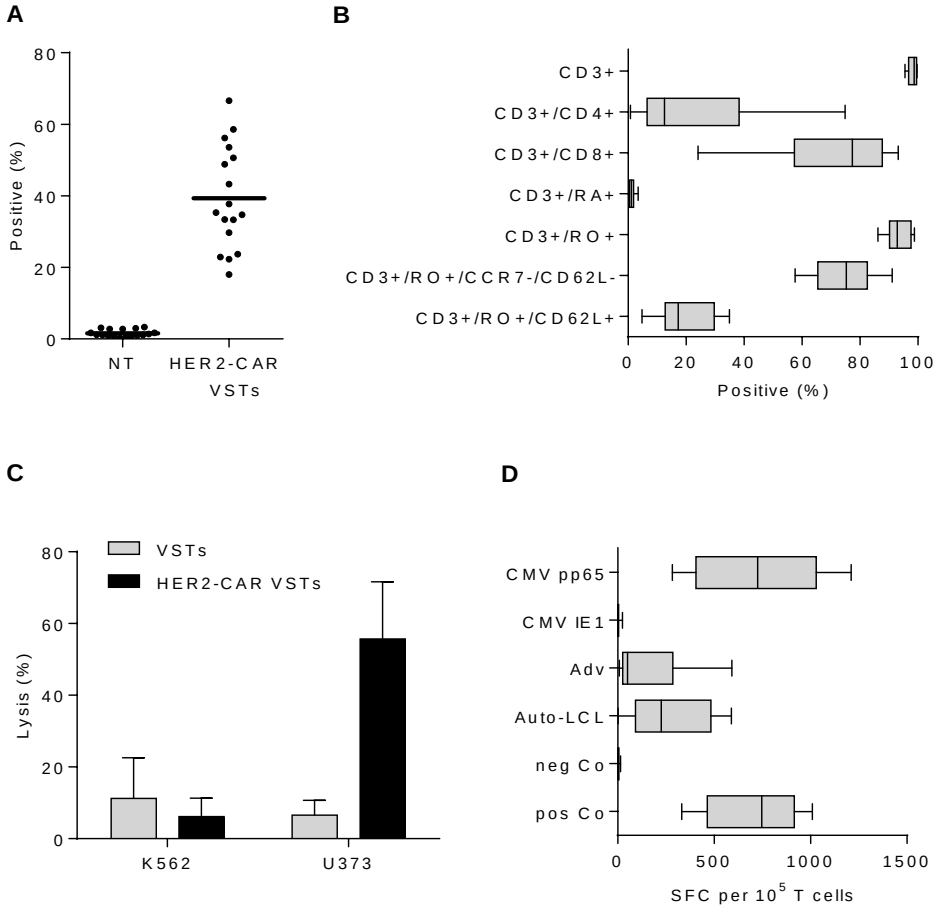
eFigure 1. Detection of HER2, CMV pp65, and CMV IE1 Expression by Immunohistochemistry in GBMs of Study Patients



Supplemental Figure 1

Immunohistochemistry was used to detect HER2, CMV pp65, and CMV IE1 expression. Results were assessed according to the following scheme: Intensity: 0 to 3+ based on positivity of control slides; Grade (cells positive): 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100%. Intensity: 1+ to 3+ based on positivity of control slides Representative images are shown (magnification 100-fold). Results for all patients are summarized in eTable 1.

eFigure 2. Characterization of HER2-CAR VST Product

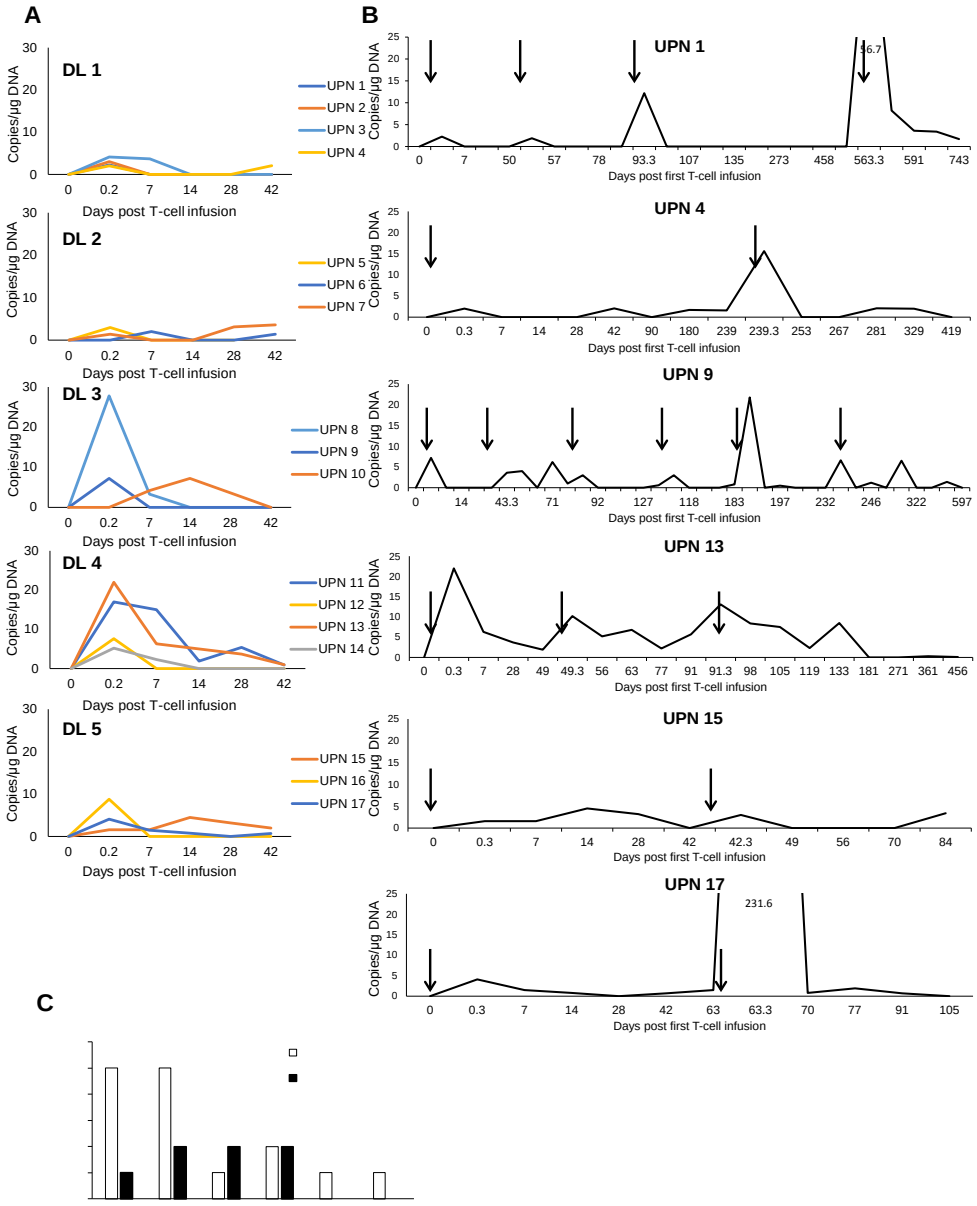


Supplemental Figure 2

(A) HER2-CAR expression on non-transduced (NT) and transduced T cells ($p<0.0001$). Individual data points and mean is shown. (B) Phenotypic analysis of HER2-CAR VST product. CM: central memory

(CD3+/CD45RO+/CD62L+); EM: effector memory (CD3+/CD45RO+/CCR7-/CD62L-). Box plot with whiskers (10 to 90 percentile) is shown. (C) Cytotoxicity assay using HER2-CAR VSTs and VSTs only as effectors and HER2-negative (K562 and HER2-positive (U373) cell lines as targets. Mean with standard deviation at an effector to target ratio of 20:1 is shown. HER2-CAR VSTs vs. VSTs only, $p<0.0001$). (D) Virus-specificity HER2-CAR VST product was determined by IFNγ Elispot assays using CMV pp65, CMV IE1, and hexon/penton (Adv) pepmixes, and auto-LCL as stimulators. PHA served as positive control (pos Co) and media as negative control (neg Co). Box plot with whiskers (10 to 90 percentile) is shown; $p<0.001$ for CMV pp65 vs neg Co, EBV vs neg Co; $p=0.005$ for Adv vs neg Co; NS for CMV IE1 vs neg Co.

eFigure 3. In vivo Persistence of HER2-CAR VSTs

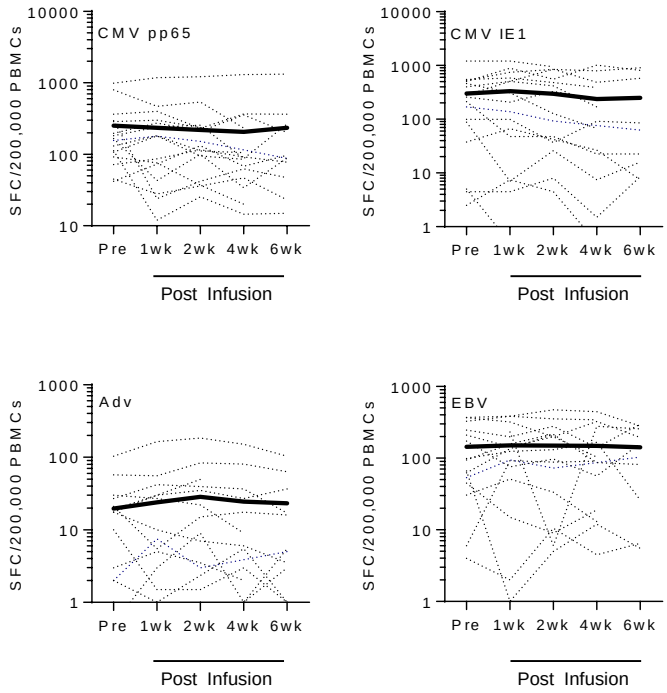


Supplemental Figure 3

In vivo persistence of HER2-CAR VSTs was detected by qPCR in the peripheral blood of patients. (A) Persistence according to dose level (DL). (B) Persistence of HER2-CAR VSTs in the peripheral blood of

patients receiving ≥2 infusions. ↓ indicates infusion of HER2-CAR VSTs. (C) HER2-CAR VSTs were detected for up to 12 months post T-cell infusion. UPN=unique patient number.

eFigure 4. Precursor Frequency of CMV-, Adv-, and EBV-Specific T-cells Post HER2-CAR VST Infusion



Supplemental Figure 4

Blood samples were obtained pre, and 1, 2, 4, and 6 week (wk) post T-cell infusion. The frequency of CMV pp65-, CMV IE1-, Adv-, and EBV-specific T cells was determined by IFNγ Elispot assays using

pepmixes (CMV pp65, CMV IE1, Adv hexon/penton) or auto-LCL as stimulators. Individual patients (dotted lines) and mean (solid line) is shown. No significant differences were observed between individual time points**.**

eReferences

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