Supplemental Methods, Data and References

Supplemental Contents

1. Supplemental Materials and Methods

Cell lines and culture

Blood samples were obtained from the Human Immunology Core of the University of Pennsylvania where peripheral blood CD4+ T cells were negatively isolated using RosetteSep Kits (Stem cell Technologies). Cells were cultured in R10 (RPMI 1640 media supplemented with 10% FCS, 100-U/ml penicillin, 100 µg/ml streptomycin sulfate, 10 mM Hepes) in a 37°C and 5% CO2 incubator. For stimulation, CD4+ T cells were cultured with activating beads coated with antibodies to CD3 and CD28 at a 1:3 cell to bead ratio. Cells were transduced with lentiviral vectors containing CAR constructs approximately 24 hrs following stimulation. T cells were monitored, kept at a concentration of $0.75x10^6$/mL and were considered rested when MCV <175. The M30 and NCI-H522 tumor lines were used in cell killing assays. The M30 cell line (53) is a mesothelial tumor derived at the University of Pennsylvania from mesothelioma tumor tissues from individual patients and was cultured in E-media (10% FCS, 1X ITES, 10 mM HEPES, 0.5 mM Na Pyruvate, 0.1 mMMEM NEAAs, 100ug/mL Pen/Strep, 1ng/mL EGF, 18 ng/mL HC, 0.1 nM T3 in RPMI) while the NCI-H522 (adenocarcinoma) was obtained from the National Cancer Institute and cultured in R10. Jurkat cell line stably transfected with a plasmid containing d2EGFP under the control of a minimal promoter bearing the NFAT consensus binding sequence (pNFAT-d2EGFP) was kindly provided by Arthur Weiss (University of California at San Francisco).

Flow cytometry and antibodies

CAR surface staining was performed in FACS buffer (PBS with 3% fetal calf serum) using biotin conjugated polyclonal antibody (Jackson ImmunoResearch). Rabbit anti-human IgG (H+L) was used for cMet IgG4, SS1 IgG4, and CD19 IgG4, while goat anti-mouse (Fab')2 was used as primary for SS1 CD8a, CD19 CD8a, and SS1 Δtail. Secondary stain for CAR was done using streptavadin-APCeFluor780 (eBioscience). Cell surface marker analysis was done using CD25 PerCp-Cy5.5 (eBioscience, clone BC96), CD70 PE (BD, clone Ki-24), PD-1 PerCP-eFluor710 (eBioscience, clone J105), CD45RO eFluor450 (eBioscience, clone UCHL1), CD27 v450 (BD, clone M-T271), CD28 FITC (eBioscience,

clone CD28.2), CD62L PE (eBioscience, clone DREG-56), CCR7 FITC (BD, clone 150503), Crtam APC (Biolegend, clone Cr24.1) and c-Met PE (R&D systems, clone 95106) at the recommended concentrations. c-Met antigen staining was done using monoclonal anti-human HGF R/c-MET-PE (R&D, clone#95106), and mesothelin expression was analyzed with primary monoclonal mouse anti-human CAK1 (Covance) at 1:50 followed by polyclonal goat anti-mouse PE (BD) at 1:100. Samples were analyzed on a LSR II (BD) and analyzed with FlowJo software (TreeStar).

PhosFlow was performed on days 6, 10 and 25. Cells were fixed using BD cytofix buffer (BD) for 10 min at 37C followed by permeabilization using BD Phosflow Perm Buffer III (BD) at 4C for 30 minutes. Cells were stained at RT for 30 min in the dark using PE anti-Erk1/2 (pT202/pY204) (BD, clone 20A), PE conjugated anti-Akt (pS473) (BD, clone M89-61), PE conjugated anti-NF-kB p65 (pS529) (BD, clone K10-895.12.50), or PE conjugated anti-S6 (pS235/pS236) (BD, clone N7-548) at the recommended concentrations. Positive controls were samples from each group stimulated for 10 min using PMA/Ionomycin prior to fixation, while negative controls were fully stimulated T cells stained using PE conjugated IgG2b kappa isotype control (BD, clone 27-35). Samples were run on a LSR II (BD Bio-sciences) and analyzed with FlowJo software (TreeStar).

Cytokine measurements

CD4+ T cells were transduced with CAR constructs as previously described. On days 6, 10, and 30 one million cells were taken from each group, pelleted, washed in R10 and plated at 1x10^6/mL in fresh media. At 24 hrs supernatant was collected and frozen at -80°C. Quantification of soluble cytokine factors was performed using Luminex bead array technology and kits purchased from Life Technologies (Invitrogen 30-plex). Assays were performed as per the manufacturer protocol with 9- point standard curve generated using a 3-fold dilution series and according to laboratory SOP. Each sample was evaluated in duplicate at 1:3 dilution; calculated % CV for the duplicate measures was in most cases less than 5% and always less than 15%. Data were acquired on a FlexMAP-3D and analyzed using XPonent 4.0 software and 5-parameter logistic regression analysis. Standard curve quantification ranges were

determined by the 80-120% (observed/expected value) range. Individual analyte quantification ranges are reported in the Figure legend.

Conditioned media transfer

Supernatant from c-Met IgG4 transduced T cell cultures was collected on day 56, filtered through a 70μm filter and frozen at -80°C in 10 mL aliquots. Day 56 media was thawed and added to unstimulated naïve CD4+ T cells in culture to reach a final concentration of 12.5%, 25%, and 50% c-Met IgG4 supernatant relative to starting media. As controls, media with and without 100IU of IL-2 was also included, as well as CD3/CD28 bead stimulated cells kept in culture with initial stimulation on day 0 and re-stimulation on day 12. Mean cell volumes were determined and cell media was readjusted every two days to maintain IL-2 concentration within control group and appropriate c-Met IgG4 media transfer ratio described above.

Vβ diversity determination

CD4+ human T cells were isolated, stimulated and transduced with c-Met IgG4 CAR as described above. Donor matched untransduced cells were stimulated and expanded simultaneously as control. Untransduced controls required two additional bead stimulations to maintain in culture. Cells were cryopreserved at D0, D13 and D34. Cells were thawed simultaneously and allowed to rest overnight. TCR Vβ analysis was performed using the IOTest Beta Mark TCR V kit (Beckman Coulter) which contains directly-conjugated antibodies specific for the following Vβ families: 1, 2, 3, 4, 5.1, 5.2, 5.3, 7.1, 7.2, 8, 9, 11, 12, 13.1, 13.2, 13.6, 14, 16, 17, 18, 20, 21.3, 22, and 23. Samples were run on a LSR II (BD) with subsequent analysis in FlowJo (TreeStar) to determine percent of total population.

Cytotoxicity assay

A mix of CD4+ and CD8+ human T cells electroporated with mRNA encoding the indicated CAR were used for in vitro killing. CD19 CD8α and c-Met IgG4 CARs were subcloned into a pGEM.64A-based vector previously described (54). The SS1 CD8α CAR mRNA was made as described (55). The replaced CAR cDNAs were confirmed by direct sequencing and linearized by *Spe*I digestion prior to RNA IVT. mScript RNA System (Epicentre, Madison, WI) was used to generate capped IVT RNA. The IVT RNA was purified using an RNeasy Mini Kit (Qiagen, Inc., Valencia, CA), and purified RNA was eluted in

RNase-free water at 1–2 mg/ml. Human T cells were stimulated by anti-CD3/CD28 beads as described (56). On day 0 the stimulated T cells were washed three times with Opti-MEM and resuspended in Opti-MEM at the final concentration of $1–3\times10^8$/ml prior to electroporation. Subsequently, the stimulated T cells were mixed with 10 μg/0.1 ml of IVT RNA (as indicated) and electroporated in a 2-mm cuvette (Harvard Apparatus BTX, Holliston, MA) using an ECM830 Electro Square Wave Porator (Harvard Apparatus BTX). Tumor lines were then harvested with trypsin and plated in a 6 well dish at 0.2x10^6/mL. 24 hours post T cell electroporation and tumor plating T cells were combined with target cells at increasing effector:target (E:T) ratios in a 6 well plate as well, alongside a no T cell control. Cells were incubated at 37°C for 18 hrs. Cells were collected after incubation, wells were re-trypsinized and washed repeatedly to collect all tumor and T cells. Cells mixtures were stained for tumor with anti-EpCAM (BD, clone EBA-1), T cells with anti-CD45 (BD, clone 2D1), and with 7-AAD (Invitrogen). Cells were resuspended in 400 uL FACS buffer containing counting beads (Invitrogen) to normalize data acquisition across samples. Samples were then filtered through a 35μm filter (BD Falcon) and put on ice for analysis. Cells were run on a LSR II (BD) and collection was performed by collecting 1500 bead events for all samples. Analysis was performed by gating on EpCAM(+), CD45(-), and 7-AAD(-) cells in FlowJo (TreeStar). Percent lysis was calculated by dividing total live cells in no T cell control group, by each experimental condition of increasing E:T ratio.

***In vivo* T cell persistence experiments**

All animal experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. NSG mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) were used for engraftment and persistence experiments. The mice were housed under specific pathogen-free conditions in microisolator cages and given unrestricted access to autoclaved food and acidified water. Animals of both sexes were used for experiments at approximately 20 weeks of age. Human CD4+ T cells were isolated, stimulated and transduced as previously described. A total of 10x10^6 cells/mouse were injected peripherally by tail vein injections of which 50% were CAR (+) in the c-Met IgG4 group. Peripheral bleeds were done after 60 days and TruCounts (BD) were done using anti-human CD45 APC-H7 staining for absolute

quantification. Samples were analyzed on a LSR II (BD Bioscience) and quantification was performed using FlowJo (TreeStar).

DNA isolation and Q-PCR analysis

Q-RT/PCR analysis: RNA was isolated from cell lines using RNAqueous RNA isolation kits (Ambion), and cDNA synthesized using iScript cDNA synthesis kits (Bio-Rad). Samples were analyzed for expression of c-met, mesothelin, and PP1B (housekeeping transcript) using ABI Taqman- based technologies and the following ABI recommended gene specific primer probe sets which span exon/intron boundaries: c-met: Hs01565584_m1*; mesothelin: HS00245879_m1*, and PP1B: Hs00168719_m1*. All amplification reactions were performed using an ABI 7500 FAST instrument (ABI-Life technologies), and established laboratory protocols. Each transcript was evaluated in triplicate. Ct values for each amplification reaction were determined using pre-established assay-specific threshold values, with a minimum of 2/3 replicates with % CV <15% required to record a Ct value. Average Ct values were calculated and reported. RQ (relative quantification) values for each transcript was determined according to the formula: $RQ=2^{-\Delta Ct}$, with $\Delta Ct=\Delta Ctsample-\Delta Ctreference$, with $\Delta Ctsample=Ctsample-Ctsample\ normalizer$ and $\Delta Ctreference=Ctreference-Ctreference\ normalizer$ (57). For all analyses of Supplementary Table S1, the ovarian carcinoma cell line OV79 (positive for both MAGE-A3) served as the reference sample. The ovarian carcinoma-derived cell line OV-79 has been previously described (58). In case of Supplementary Figure 6, untransduced T cells served as a reference sample.

Imunohistochemistry

Tumor tissues were embedded in OCT (Tissue-Tek) and 10μm frozen sections were immunostained with anti-CD3 (Thermo Scientific) antibodies and counterstained with Hematoxylin. Staining was performed by the Pathology Core Laboratories at The Children's Hospital of Philadelphia.

2. Supplemental Tables

Table S1. Expression of c-Met and Mesothelin mRNA in activated CD4 T Cells.

SAMPLE INFORMATION		PP1B	c-MET		Mesothelin	
CELL LINE	Timepoint of harvest	Ct	Ct	RQ	Ct	RQ
OV79 (Reference)	none	19.4	23.6	1	23.7	1
HD340 resting	Day 0	27.6	37.1	0.023	ND	-
HD340 c-Met IgG4	Day 6	22.2	32.8	0.011	37.9	0.0003
HD340 c-Met IgG4	Day 11	21.7	33.1	0.006	38.7	0.0001
HD340 c-Met IgG4	Day 24	23.9	34.0	0.016	39.5	0.0004
HD306 resting	Day 0	26.7	34.8	0.066	39.2	0.0036
HD306 mock	Day 6	22.9	33.5	0.011	38.9	0.0003
HD306 mock	Day 11	22.5	34.2	0.005	37.5	0.0006
HD306 mock	Day 24	23.9	36.7	0.002	ND	-
HD256 resting	Day 0	26.9	39.3	0.003	ND	-
HD256 c-Met IgG4	Day 6	23.0	34.6	0.006	37.4	0.0009
HD256 c-Met IgG4	Day 11	21.4	33.2	0.005	37.3	0.0003

CD4+ T cells were cultured as described in legend to Figures 2 and 6, harvested and frozen viably. RNA was isolated from frozen cells, cDNA synthesized, and Q-PCR analysis performed at the indicated time points. Relative Quantification (RQ) values for each sample were determined relative to OV79, a reference tumor cell line known to express c-Met and mesothelin, and using PP1B as the housekeeping gene. HD, healthy donor; ND: not detected.

Table S2. Genes upregulated in CD4+ T cells of healthy human adults expressing c-Met IgG4 continuous CAR compared to CD19 CD8α non-continuous CAR on day 11.

Gene Name	Gene Symbol	RefSeq or Affymetrix ID	p-value	Fold Change
nuclear factor, interleukin 3 regulated	NFIL3	NM_005384	6.65E-09	5.03
Rho GTPase activating protein 11A	ARHGAP11A	NM_014783	2.60E-07	5.04
germ cell associated 2 (haspin)	GSG2	NM_031965	3.44E-08	5.04
ubiquitin-conjugating enzyme E2T (putative)	UBE2T	NM_014176	2.73E-11	5.10
replication factor C (activator 1) 4, 37kDa	RFC4	NM_002916	6.99E-10	5.11
breast cancer 2, early onset	BRCA2	NM_000059	1.26E-10	5.11
fatty acid desaturase 1	FADS1	NM_013402	1.88E-10	5.13
minichromosome maintenance complex component 4	MCM4	NM_005914	3.85E-11	5.14
kinesin family member 20B	KIF20B	NM_016195	9.59E-07	5.19
annexin A3	ANXA3	NM_005139	2.29E-04	5.20
Fanconi anemia, complementation group I	FANCI	NM_001113378	1.21E-12	5.21
ATPase family, AAA domain containing 5	ATAD5	NM_024857	2.45E-09	5.21
histone cluster 2, H2ab	HIST2H2AB	NM_175065	2.67E-07	5.21
nucleolar and spindle associated protein 1	NUSAP1	NM_016359	3.67E-08	5.22
cell division cycle associated 3	CDCA3	NM_031299	1.06E-10	5.22
natural killer cell group 7 sequence	NKG7	NM_005601	2.73E-05	5.24
MLF1 interacting protein	MLF1IP	NM_024629	1.60E-08	5.25
cyclin-dependent kinase 2	CDK2	NM_001798	5.38E-11	5.25
extra spindle pole bodies homolog 1 (S. cerevisiae)	ESPL1	NM_012291	4.50E-11	5.27
DNA replication helicase 2 homolog (yeast)	DNA2	NM_001080449	2.85E-10	5.28
guanine nucleotide binding protein (G protein), alpha 15 (Gq class)	GNA15	NM_002068	3.11E-08	5.30
solute carrier family 26, member 4	SLC26A4	NM_000441	7.25E-09	5.32
LIM domain and actin binding 1	LIMA1	NM_001113546	4.21E-11	5.42
histone cluster 1, H2al	HIST1H2AL	NM_003511	2.21E-10	5.44
chemokine (C-C motif) receptor 2	CCR2	NM_001123041	1.16E-06	5.45
ELOVL family member 6, elongation of long chain fatty acids	ELOVL6	NM_024090	1.23E-08	5.45
kinesin family member C1	KIFC1	NM_002263	1.47E-08	5.46
Nedd4 family interacting protein 2	NDFIP2	NM_019080	6.67E-10	5.47
dihydrofolate reductase	DHFR	NM_000791	3.63E-07	5.51
CDC28 protein kinase regulatory subunit 1B	CKS1B	NM_001826	1.05E-08	5.51
replication factor C (activator 1) 3, 38kDa	RFC3	NM_002915	4.02E-10	5.51
gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)	GGH	NM_003878	5.41E-09	5.52
transmembrane protein 106C	TMEM106C	NM_001143842	2.66E-08	5.53
cell division cycle associated 5	CDCA5	NM_080668	1.48E-08	5.54
centromere protein A	CENPA	NM_001809	1.21E-11	5.55
protein regulator of cytokinesis 1	PRC1	NM_003981	1.08E-10	5.55
CHK1 checkpoint homolog (S. pombe)	CHEK1	NM_001274	1.23E-09	5.58

Gene Name	Gene Symbol	RefSeq or Affymetrix ID	p-value	Fold Change
origin recognition complex, subunit 1	ORC1	NM_004153	1.74E-15	5.60
CDC28 protein kinase regulatory subunit 1B	CKS1B	NM_001826	8.55E-09	5.64
dihydrofolate reductase pseudogene	LOC1720	NR_033423	1.31E-06	5.68
interferon, gamma	IFNG	NM_000619	6.16E-08	5.71
interleukin 2 receptor, alpha	IL2RA	NM_000417	7.32E-12	5.76
ATPase, class I, type 8B, member 4	ATP8B4	NM_024837	1.22E-09	5.77
CD180 molecule	CD180	NM_005582	1.24E-04	5.79
chemokine (C-C motif) receptor 2	CCR2	NM_001123396	1.48E-07	5.85
breast cancer 1, early onset	BRCA1	NR_027676	2.27E-09	5.86
hypothetical LOC151009	LOC151009	AK095678	5.30E-09	5.93
coiled-coil domain containing 99	CCDC99	NM_017785	1.07E-07	5.95
citron (rho-interacting, serine/threonine kinase 21)	CIT	NM_007174	3.48E-13	5.96
oncostatin M	OSM	NM_020530	1.30E-08	6.00
solute carrier family 43, member 3	SLC43A3	NM_017611	1.20E-10	6.01
chromosome 11 open reading frame 82	C11orf82	NM_145018	1.81E-08	6.01
interferon, gamma-inducible protein 30	IFI30	NM_006332	3.78E-04	6.02
histone cluster 1, H3b	HIST1H3B	NM_003537	3.45E-07	6.03
BRCA1 interacting protein C-terminal helicase 1	BRIP1	NM_032043	4.69E-09	6.11
KDEL (Lys-Asp-Glu-Leu) containing 1	KDELC1	NM_024089	1.03E-08	6.16
centromere protein F, 350/400kDa (mitosin)	CENPF	NM_016343	7.84E-11	6.19
RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)	RAD51	NM_002875	3.01E-10	6.20
WD repeat and HMG-box DNA binding protein 1	WDHD1	NM_007086	2.71E-09	6.23
DEP domain containing 1B	DEPDC1B	NM_018369	2.17E-12	6.24
centromere protein N	CENPN	NM_001100624	4.70E-09	6.29
X-ray repair complementing defective repair in Chinese hamster cells 2	XRCC2	NM_005431	1.44E-09	6.32
solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	SLC7A11	NM_014331	5.74E-05	6.32
purinergic receptor P2Y, G-protein coupled, 14	P2RY14	NM_014879	1.32E-08	6.34
exonuclease 1	EXO1	NM_130398	2.84E-11	6.37
phosphoglycerate dehydrogenase	PHGDH	NM_006623	4.54E-11	6.37
chemokine (C-C motif) ligand 4	CCL4	NM_002984	8.33E-06	6.39
interleukin 18 receptor accessory protein	IL18RAP	NM_003853	1.32E-06	6.40
granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1	GZMB	NM_004131	9.23E-07	6.43
spindle and kinetochore associated complex subunit 3	SKA3	NM_145061	8.97E-07	6.47
histone cluster 1, H2bm	HIST1H2BM	NM_003521	1.58E-07	6.49
nei endonuclease VIII-like 3 (E. coli)	NEIL3	NM_018248	4.64E-09	6.53
RAB27B, member RAS oncogene family	RAB27B	NM_004163	1.01E-08	6.55
epithelial membrane protein 1	EMP1	NM_001423	6.73E-07	6.60
v-myb myeloblastosis viral oncogene homolog (avian)-like 2	MYBL2	NM_002466	3.47E-09	6.65

Gene Name	Gene Symbol	RefSeq or Affymetrix ID	p-value	Fold Change
KIAA1524, Protein CIP2A also known as cancerous inhibitor of PP2A (CIP2A)	KIAA1524	NM_020890	1.35E-10	6.68
hematopoietic prostaglandin D synthase	HPGDS	NM_014485	1.80E-07	6.72
interleukin 18 receptor 1	IL18R1	NM_003855	8.92E-08	6.75
minichromosome maintenance complex component 8	MCM8	NM_032485	1.11E-11	6.75
non-SMC condensin II complex, subunit G2	NCAPG2	NM_017760	1.27E-10	6.76
thyroid hormone receptor interactor 13	TRIP13	NM_004237	1.91E-13	6.79
interleukin 21	IL21	NM_021803	3.95E-07	6.81
chromosome 15 open reading frame 42	C15orf42	NM_152259	2.01E-11	6.82
E2F transcription factor 8	E2F8	NM_024680	2.50E-12	6.84
NIMA (never in mitosis gene a)-related kinase 2	NEK2	NM_002497	4.79E-09	6.89
minichromosome maintenance complex component 10	MCM10	NM_182751	5.93E-11	7.01
ribonucleotide reductase M2	RRM2	NM_001165931	1.42E-07	7.02
histone cluster 1, H2bb	HIST1H2BB	NM_021062	8.90E-07	7.04
cell division cycle associated 2	CDCA2	NM_152562	3.54E-11	7.13
Holliday junction recognition protein	HJURP	NM_018410	2.17E-12	7.14
proline rich 11	PRR11	NM_018304	7.94E-09	7.15
polymerase (DNA directed), epsilon 2 (p59 subunit)	POLE2	NM_002692	3.57E-10	7.18
chromosome 4 open reading frame 21	C4orf21	NM_018392	3.41E-09	7.18
polymerase (DNA directed), theta	POLQ	NM_199420	1.82E-10	7.19
histone cluster 1, H1b	HIST1H1B	NM_005322	3.60E-08	7.20
budding uninhibited by benzimidazoles 1 homolog beta (yeast)	BUB1B	NM_001211	1.98E-09	7.21
family with sequence similarity 54, member A	FAM54A	NM_001099286	5.31E-11	7.27
epithelial cell transforming sequence 2 oncogene, ARHGEF31	ECT2	NM_018098	5.05E-09	7.32
cyclin A1	CCNA1	NM_003914	3.86E-09	7.41
kinesin family member 4A	KIF4A	NM_012310	1.42E-08	7.42
chromosome 4 open reading frame 46	C4orf46	NM_001008393	6.31E-08	7.43
interleukin 1, alpha	IL1A	NM_000575	9.74E-09	7.55
SCL/TAL1 interrupting locus	STIL	NM_001048166	1.48E-08	7.56
aurora kinase A	AURKA	NM_198433	8.44E-08	7.64
cyclin B1	CCNB1	NM_031966	1.75E-09	7.64
ASF1 anti-silencing function 1 homolog B (S. cerevisiae)	ASF1B	NM_018154	2.42E-10	7.65
forkhead box M1	FOXM1	NM_202002	6.65E-10	7.74
stearoyl-CoA desaturase (delta-9-desaturase)	SCD	NM_005063	2.27E-12	7.76
cell division cycle 45 homolog (S. cerevisiae)	CDC45	NM_001178010	4.81E-11	7.82
diaphanous homolog 3 (Drosophila)	DIAPH3	NM_001042517	2.67E-11	7.89
kinesin family member 23	KIF23	NM_138555	3.97E-08	7.94
sperm associated antigen 5	SPAG5	NM_006461	1.19E-11	7.95
polo-like kinase 4	PLK4	NM_014264	7.19E-11	8.00

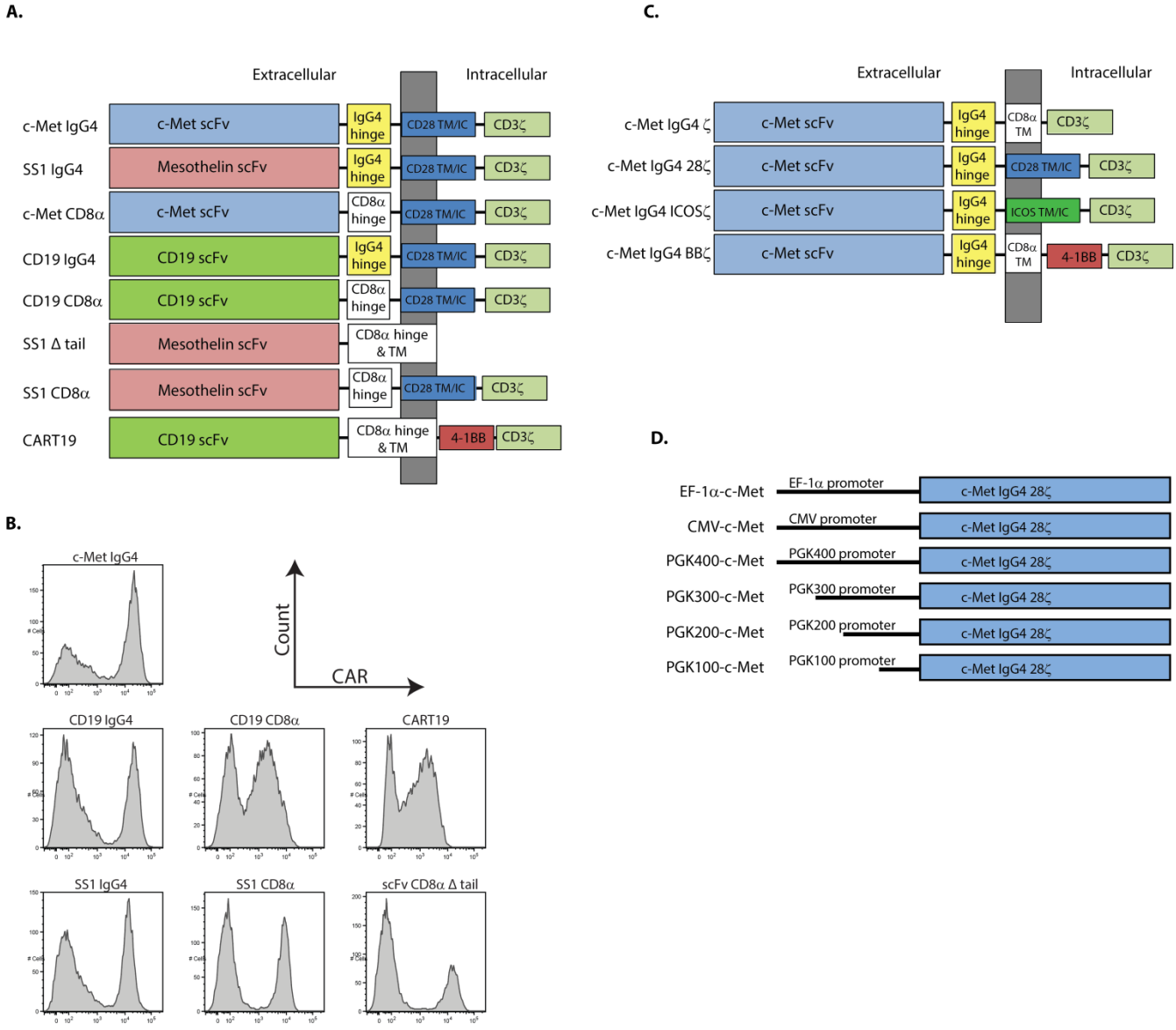
Gene Name	Gene Symbol	RefSeq or Affymetrix ID	p-value	Fold Change
kinesin family member 11	KIF11	NM_004523	4.17E-09	8.03
cell division cycle associated 8	CDCA8	NM_018101	3.48E-11	8.04
KIAA0101, PCNA-associated factor	KIAA0101	NM_014736	6.75E-08	8.07
meiotic nuclear divisions 1 homolog (S. cerevisiae)	MND1	NM_032117	2.40E-08	8.09
kinesin family member 2C	KIF2C	NM_006845	1.08E-10	8.11
TPX2, microtubule-associated, homolog (Xenopus laevis)	TPX2	NM_012112	6.41E-11	8.15
KI-678, antigen identified by monoclonal antibody Ki-67	MKI67	NM_002417	2.81E-10	8.16
claspin	CLSPN	NM_022111	4.84E-09	8.21
kinesin family member 18A	KIF18A	NM_031217	3.37E-08	8.22
non-SMC condensin I complex, subunit H	NCAPH	NM_015341	4.27E-09	8.23
defective in sister chromatid cohesion 1 homolog (S. cerevisiae)	DSCC1	NM_024094	1.06E-09	8.26
shugoshin-like 2 (S. pombe)	SGOL2	NM_152524	4.10E-09	8.46
apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	APOBEC3B	NM_004900	5.40E-09	8.49
phosphatidic acid phosphatase type 2A	PPAP2A	NM_003711	1.13E-12	8.49
cyclin A2	CCNA2	NM_001237	1.06E-09	8.64
kinesin family member 20A	KIF20A	NM_005733	1.99E-09	8.88
anillin, actin binding protein	ANLN	NM_018685	3.60E-09	8.93
thymidylate synthetase	TYMS	NM_001071	8.77E-10	8.98
topoisomerase (DNA) II alpha 170kDa	TOP2A	NM_001067	2.21E-09	9.03
histone cluster 1, H3f	HIST1H3F	NM_021018	1.11E-06	9.08
GINS complex subunit 2 (Psf2 homolog)	GINS2	NM_016095	2.85E-10	9.09
family with sequence similarity 111, member B	FAM111B	NM_198947	7.28E-07	9.13
chemokine (C-C motif) receptor 1	CCR1	NM_001295	7.58E-09	9.19
cytoskeleton associated protein 2-like	CKAP2L	NM_152515	1.86E-11	9.23
centromere protein E, 312kDa	CENPE	NM_001813	1.99E-08	9.27
asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	ASPM	NM_018136	2.06E-10	9.28
cyclin-dependent kinase inhibitor 3	CDKN3	NM_005192	1.53E-08	9.33
polo-like kinase 1	PLK1	NM_005030	6.58E-12	9.54
TTK protein kinase, hMps1	TTK	NM_003318	4.75E-10	9.55
cancer susceptibility candidate 5	CASC5	NM_170589	1.09E-08	9.78
spindle and kinetochore associated complex subunit 1	SKA1	NM_001039535	3.16E-10	9.86
DEP domain containing 1	DEPDC1	NM_001114120	2.52E-09	9.86
non-SMC condensin I complex, subunit G	NCAPG	NM_022346	6.57E-10	9.95
budding uninhibited by benzimidazoles 1 homolog (yeast)	BUB1	NM_004336	5.75E-11	10.12
RAD51 associated protein 1	RAD51AP1	NM_001130862	2.43E-10	10.14
kinesin family member 14	KIF14	NM_014875	1.92E-10	10.15
colony stimulating factor 2 (granulocyte-macrophage)	CSF2	NM_000758	1.58E-08	10.28

Gene Name	Gene Symbol	RefSeq or Affymetrix ID	p-value	Fold Change
fatty acid desaturase 2	FADS2	NM_004265	1.84E-09	10.31
heparin-binding EGF-like growth factor	HBEGF	NM_001945	2.31E-09	10.31
GINS complex subunit 1 (Psf1 homolog)	GINS1	NM_021067	5.35E-12	10.31
shugoshin-like 1 (S. pombe)	SGOL1	NM_001012410	1.89E-09	10.47
cell division cycle 20 homolog (S. cerevisiae)	CDC20	NM_001255	1.32E-10	10.50
family with sequence similarity 72, member D	FAM72D	AB096683	5.52E-11	10.74
maternal embryonic leucine zipper kinase	MELK	NM_014791	1.92E-10	10.75
interleukin 17 receptor B	IL17RB	NM_018725	5.42E-08	10.85
family with sequence similarity 72, member D	FAM72D	AB096683	2.82E-10	10.91
kinesin family member 15	KIF15	NM_020242	1.64E-10	10.99
interleukin 3 (colony-stimulating factor, multiple)	IL3	NM_000588	4.31E-10	11.00
family with sequence similarity 72, member D	FAM72D	AB096683	3.17E-10	11.21
centromere protein I	CENPI	NM_006733	9.64E-11	11.32
NUF2, NDC80 kinetochore complex component, homolog (S. cerevisiae)	NUF2	NM_145697	1.20E-10	11.33
hyaluronan-mediated motility receptor (RHAMM)	HMMR	NM_001142556	3.12E-12	11.76
PDZ binding kinase	PBK	NM_018492	1.35E-09	12.06
SHC SH2-domain binding protein 1	SHCBP1	NM_024745	3.84E-11	12.24
centrosomal protein 55kDa	CEP55	NM_018131	1.93E-09	12.29
cell division cycle 6 homolog (S. cerevisiae)	CDC6	NM_001254	7.19E-11	12.52
SPC25, NDC80 kinetochore complex component, homolog (S. cerevisiae)	SPC25	NM_020675	5.68E-10	12.74
leukemia inhibitory factor (cholinergic differentiation factor)	LIF	NM_002309	8.95E-10	12.89
cyclin E2	CCNE2	NM_057749	6.32E-11	13.30
denticleless homolog (Drosophila)	DTL	NM_016448	1.59E-12	13.39
discs, large (Drosophila) homolog-associated protein 5	DLGAP5	NM_014750	1.11E-09	13.69
establishment of cohesion 1 homolog 2 (S. cerevisiae)	ESCO2	NM_001017420	2.92E-11	13.84
cyclin B2	CCNB2	NM_004701	5.37E-10	14.57
granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	GZMA	NM_006144	7.81E-12	15.62
cyclin-dependent kinase 1	CDK1	NM_001786	1.73E-09	19.22
family with sequence similarity 72, member D	FAM72D	AB096683	1.69E-08	19.52
dual specificity phosphatase 6	DUSP6	NM_001946	1.34E-09	22.97
chemokine (C-X-C motif) ligand 10	CXCL10	NM_001565	1.61E-05	25.53
interleukin 13	IL13	NM_002188	4.92E-12	42.92
prostaglandin I2 (prostacyclin) synthase	PTGIS	NM_000961	8.14E-12	44.57

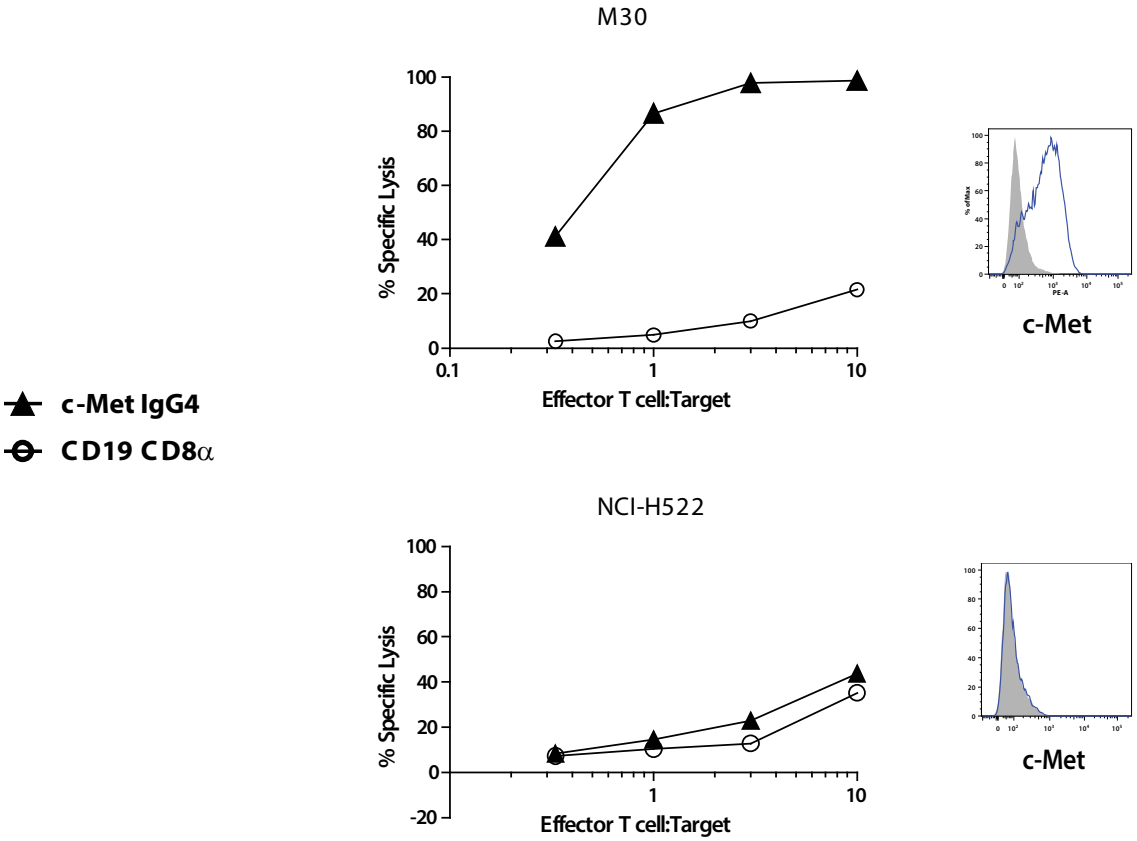
Table S3. Genes downregulated in CD4+ T cells of healthy human adults expressing c-Met IgG4 continuous CAR compared to CD19 CD8α non-continuous CAR on day 11.

Gene Name	Gene Symbol	RefSeq or Affymetrix ID	p-value	Fold Change
---	---	8168079	3.08E-04	-5.01
kelch-like 24 (Drosophila)	KLHL24	NM_017644	3.09E-10	-5.01
---	---	8107857	5.02E-04	-5.07
small nucleolar RNA, C/D box 116-4	SNORD116-4	NR_003319	1.03E-06	-5.18
small nucleolar RNA, C/D box 116-8	SNORD116-8	NR_003323	7.66E-08	-5.19
small nucleolar RNA, H/ACA box 1	SNORA1	NR_003026	4.83E-04	-5.24
small nucleolar RNA, H/ACA box 10	SNORA10	NR_002327	5.19E-05	-5.29
adenylate kinase 5	AK5	NM_174858	1.30E-07	-5.38
odz, odd Oz/ten-m homolog 1(Drosophila)	ODZ1	NM_001163278	3.17E-06	-5.41
small nucleolar RNA, C/D box 6	SNORD6	NR_003036	7.26E-04	-5.42
LY9, lymphocyte antigen 9	LY9	NM_002348	2.40E-07	-5.45
killer cell lectin-like receptor subfamily B, member 1	KLRB1	NM_002258	1.03E-06	-5.46
small Cajal body-specific RNA 5	SCARNA5	NR_003008	1.23E-05	-5.53
---	---	8135943	5.12E-04	-5.73
small nucleolar RNA, H/ACA box 52	SNORA52	NR_002585	5.27E-05	-5.81
Fas apoptotic inhibitory molecule 3	FAIM3	NM_005449	8.07E-11	-5.89
transmembrane protein 63A	TMEM63A	NM_014698	2.22E-08	-5.95
programmed cell death 4 (neoplastic transformation inhibitor)	PDCD4	NM_145341	4.02E-10	-6.21
small nucleolar RNA, H/ACA box 60	SNORA60	NR_002986	1.66E-04	-6.30
---	---	8016213	1.73E-04	-6.37
---	---	7938625	8.47E-04	-6.55
ribonuclease P RNA component H1	RPPH1	NR_002312	2.86E-06	-6.61
---	---	7958273	1.11E-07	-6.91
---	---	8031931	9.24E-04	-6.96
G protein-coupled receptor 155	GPR155	NM_001033045	2.78E-10	-7.28
phosphoinositide-3-kinase interacting protein 1	PIK3IP1	NM_052880	2.07E-09	-7.43
small nucleolar RNA, H/ACA box 42	SNORA42	NR_002974	2.76E-05	-7.46
t-complex 11 (mouse)-like 2	TCP11L2	NM_152772	1.66E-09	-7.58
small Cajal body-specific RNA 4	SCARNA4	NR_003005	2.37E-04	-7.68
---	---	8050350	3.26E-04	-8.50
RNA, U4 small nuclear 2	RNU4-2	NR_003137	1.31E-04	-8.63
V-set and immunoglobulin domain containing 1	VSIG1	NM_001170553	7.85E-09	-8.72
small Cajal body-specific RNA 6	SCARNA6	NR_003006	1.60E-05	-9.36
---	---	8073680	4.26E-04	-9.59
EPH receptor A4	EPHA4	NM_004438	5.16E-10	-10.77
NEL-like 2 (chicken)	NELL2	NM_006159	7.47E-09	-12.65

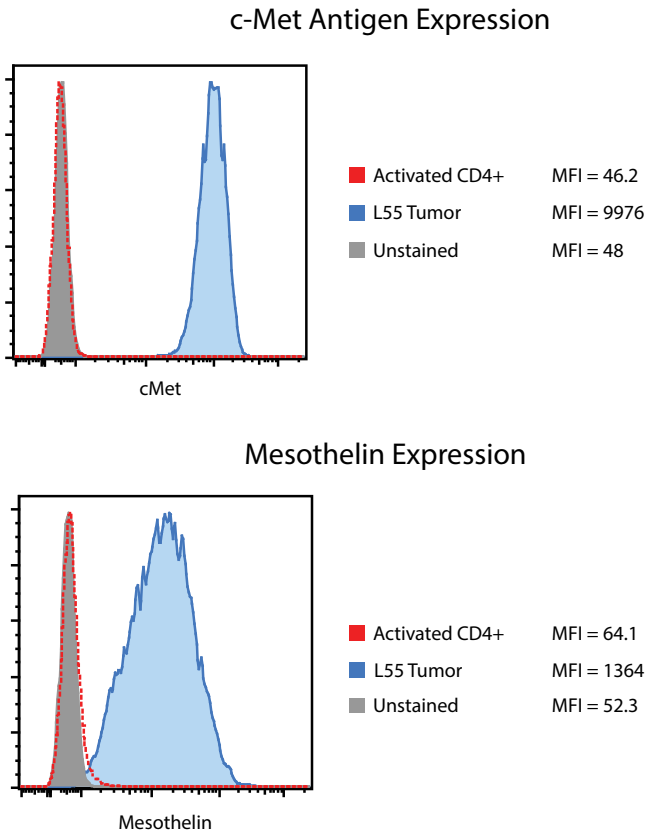
3. Supplemental Figures and Legends



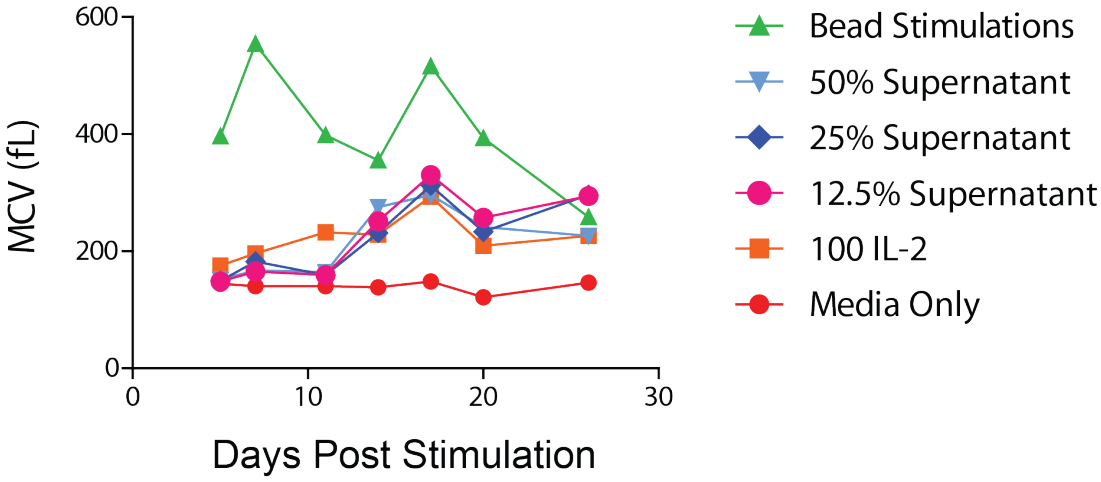
Supplemental Figure 1. Chimeric antigen receptor constructs and relative expression levels. A) Representation of CAR constructs depicting the various scFv, hinge regions, transmembrane and cytosolic domains. **B**) The surface expression of each CAR construct in (A) was analyzed 6 days following lentiviral transduction to quantify relative expression levels. CAR expression was determined as described in Methods. **C**) CAR constructs used in main text figures 1 to 5. **D**) CAR constructs with promoter variations used in the experiments shown in main text figures 6 and 7.



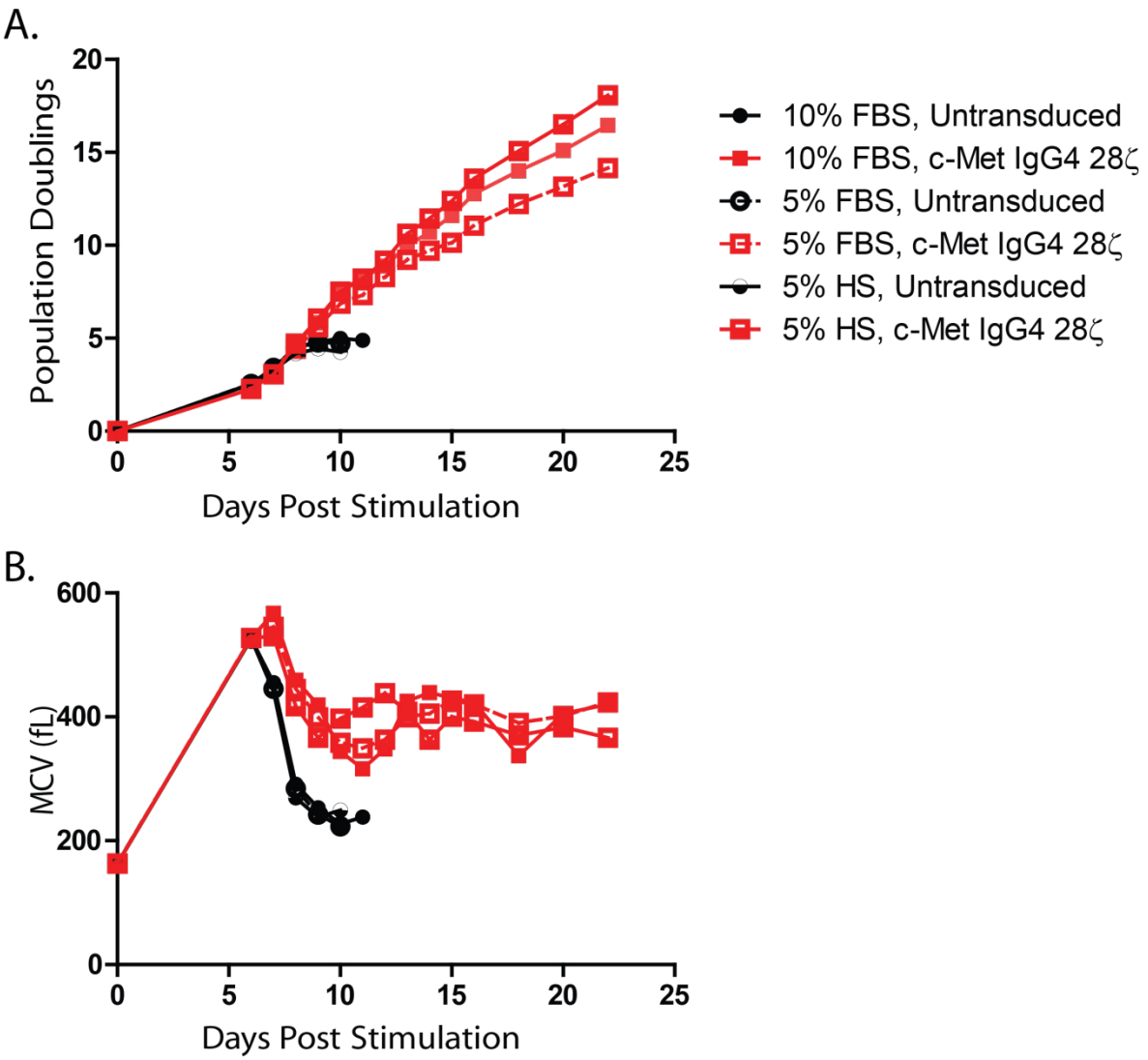
Supplemental Figure 2. CAR T cells with constitutive proliferation retain specific cytotoxicity. The M30 tumor line (endogenous expression of c-Met), and the NCI-H522 tumor line (lacking c-Met expression) were cultured at the indicated effector to target ratio with c-Met IgG4 CAR T cells. CD19 CD8α CAR T cells were used as specificity controls to exclude allogeneic effects. Inset boxes: c-Met expression on M30 and NCI-H522.



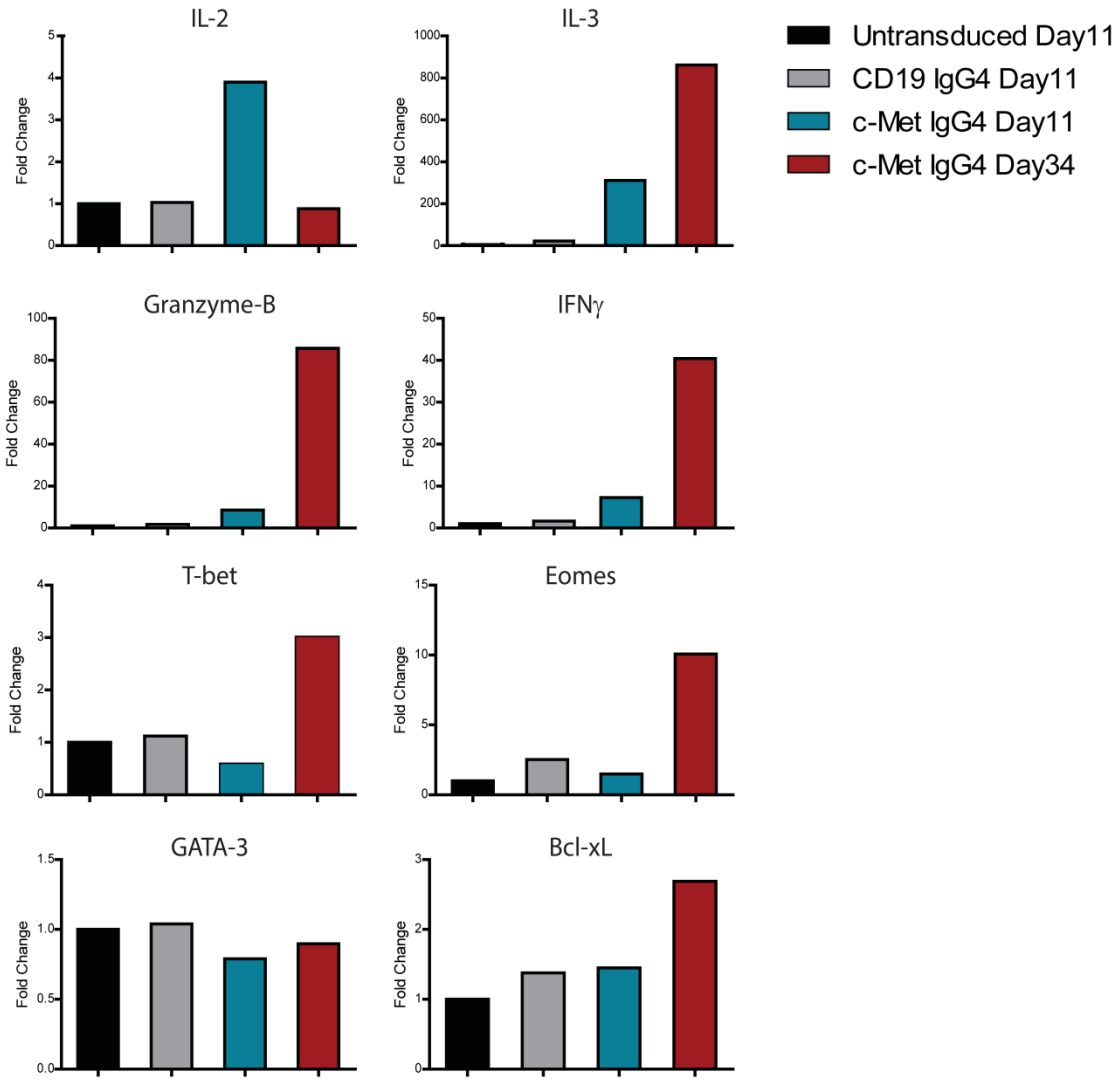
Supplemental Figure 3. c-Met and mesothelin expression are not detected on human CD4+ T cells. Samples were compared to L55, a non-small cell lung tumor cell line, as well as unstained CD4+ T cells. Following activation, human CD4+ T cells and tumor lines were stained for c-Met (PE) or mesothelin (PE). Histograms depict unstained activated CD4+ T cells (grey), activated CD4+ T cells (red), and the L55 tumor stained for described antigen. The mean fluorescence intensity (MFI) is indicated.



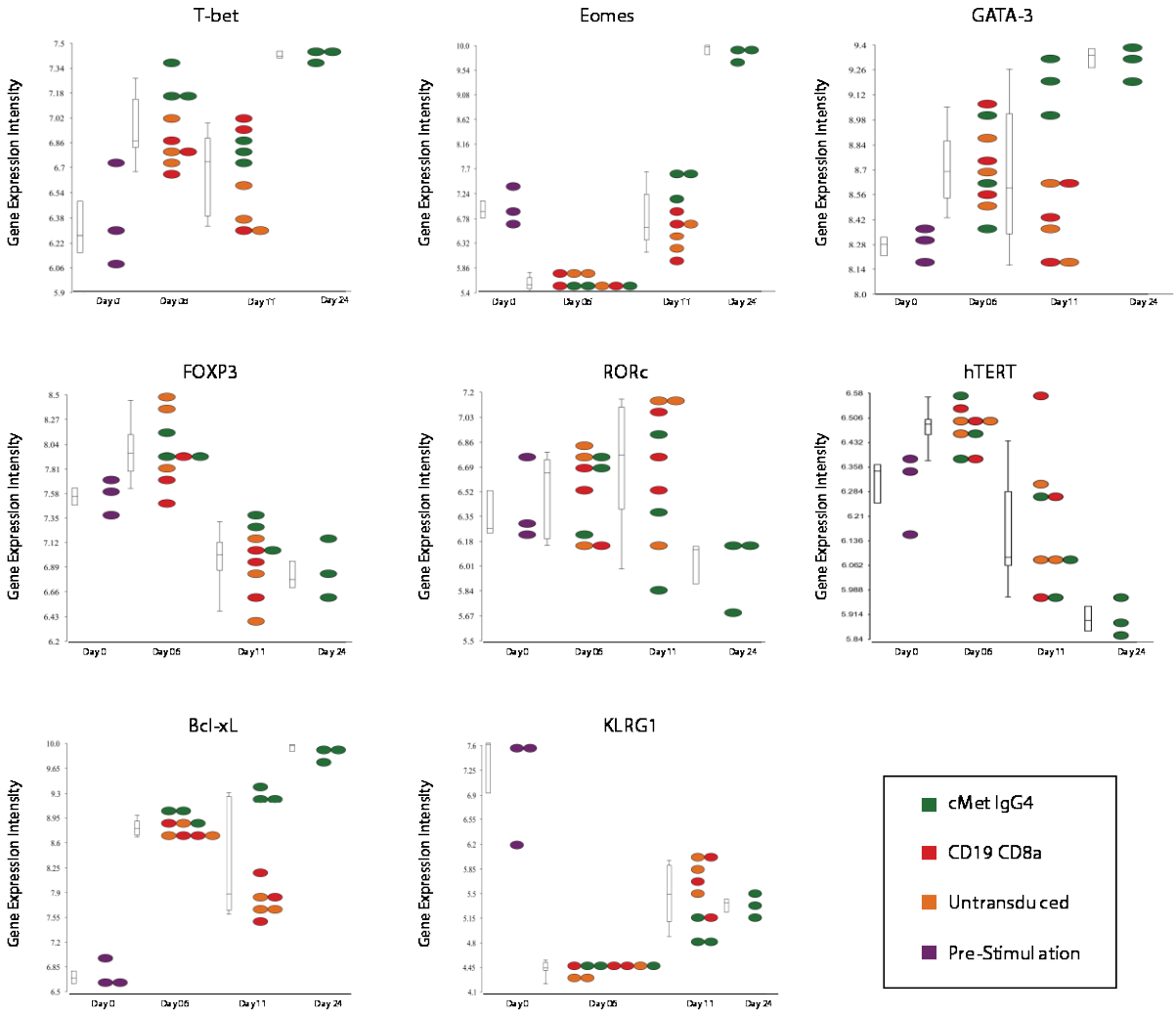
Supplemental Figure 4. Supernatant from CARs displaying the growth phenotype induces activation of naïve unstimulated T cells. Culture supernatant from c-Met IgG4 CAR T cell culture harvested on day 56 of culture was added to unstimulated naïve CD4+ T cells at a final concentration of 12.5%, 25%, or 50% c-Met IgG4 supernatant relative to starting media. As controls, media with and without 100IU of IL-2 were also included, as well as anti-CD3/CD28 bead stimulated untransduced T cells kept in culture with initial stimulation on day 0 and re-stimulation on day 12. MCVs were determined and cell media was added every two days to maintain the supernatant concentration and IL-2 concentration within control group as described above.



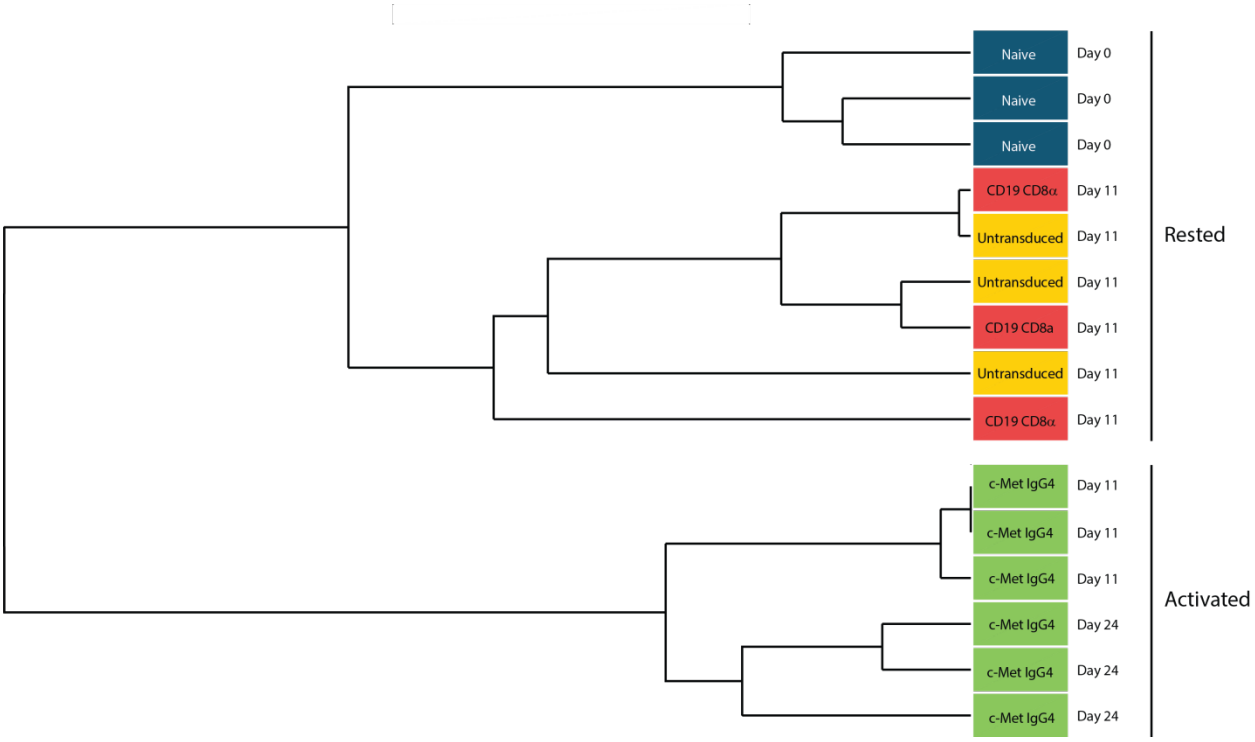
Supplemental Figure 5. The role of fetal bovine serum in the constitutive growth of c-Met IgG4 28ζ CAR T cells. In vitro proliferation of CD4+ T cells following 5 days of αCD3/CD28 coated magnetic bead stimulation and lentiviral transduction. All cells were initially activated and propagated in 10% fetal bovine serum (FBS) containing culture media. 6 days after activation, cells were separately grown in media containing 10% FBS, 5% FBS or 5% human serum (HS). Population doubling (**A**) and mean cell volume (MCV) (**B**) were determined.



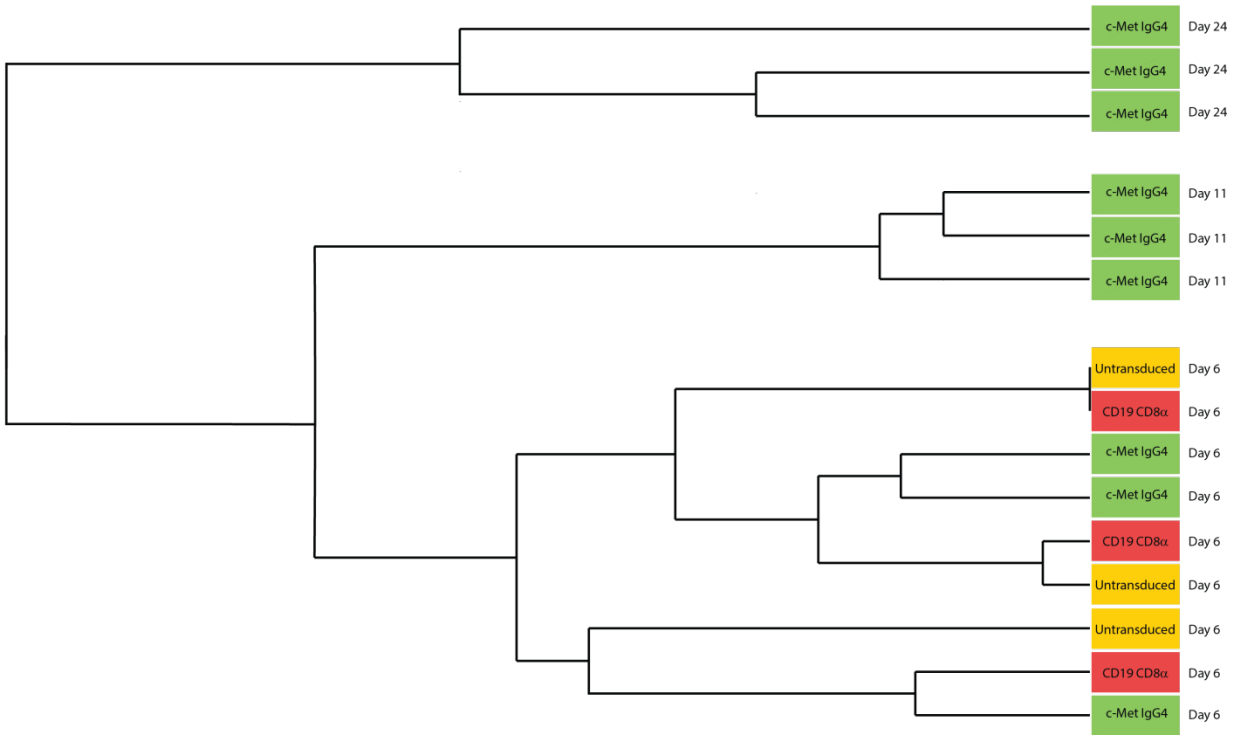
Supplemental Figure 6A. CARs with a constitutive growth phenotype display a unique gene signature. Real-time PCR analysis comparing expression of genes between CD19 IgG4 (gray), c-Met IgG4 (day11 – blue, day34 – red) transduced and untransduced (black) T cells at noted timepoints. Fold change are plotted relative to expression level of untransduced T cells. The design of the CAR constructs is shown in Supplemental Figure 1.



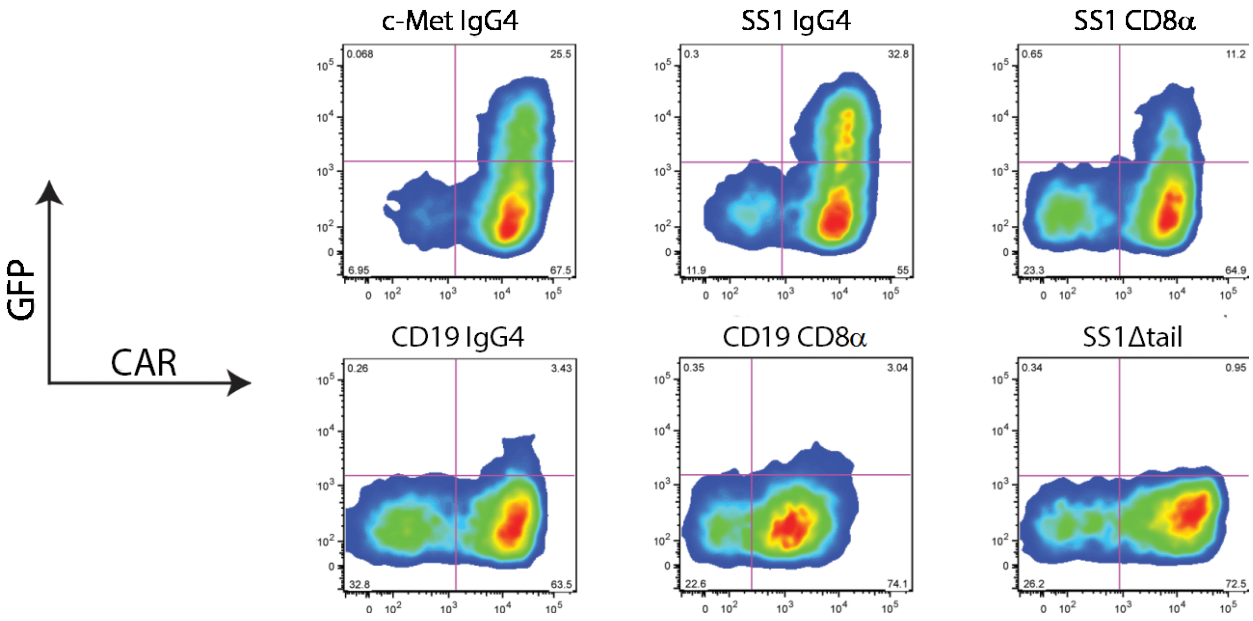
Supplemental Figure 6B. CAR T cells with a constitutive growth phenotype display distinct transcription factors. Expression of genes important for T cell polarization, growth and survival: T-bet, Eomes, GATA-3, RORc, FoxP3, Bcl-xL, KLRG1, and hTERT. Normalized absolute log(2) gene expression intensities are plotted. Data is compilation of normal donor triplicates analyzed prior to stimulation (purple), and on days 6, 11 and 24; only the c-Met IgG4 culture is analyzed on day 24 because the other cultures were terminated due to cell death. Each dot denotes a single donor within each time point expressing either the c-Met IgG4 CAR (green), CD19 CD8α CAR (red), or untransduced control (orange). Box plots representing upper 75th and lower 25th percentile with median. Whiskers denote upper 90th and lower 10th percentile. Comparison of c-Met IgG4 CAR (green) vs CD19 CD8α CAR (red) on day 11 by ANOVA: T-bet ($p = 0.888$); Eomes ($p = 0.003$); GATA-3 ($p < 0.001$); FoxP3 ($p = 0.122$); RORc ($p = 0.089$); KLRG1 ($P = 0.076$); hTERT ($p = 0.405$); and Bcl-xL ($p < 0.001$).



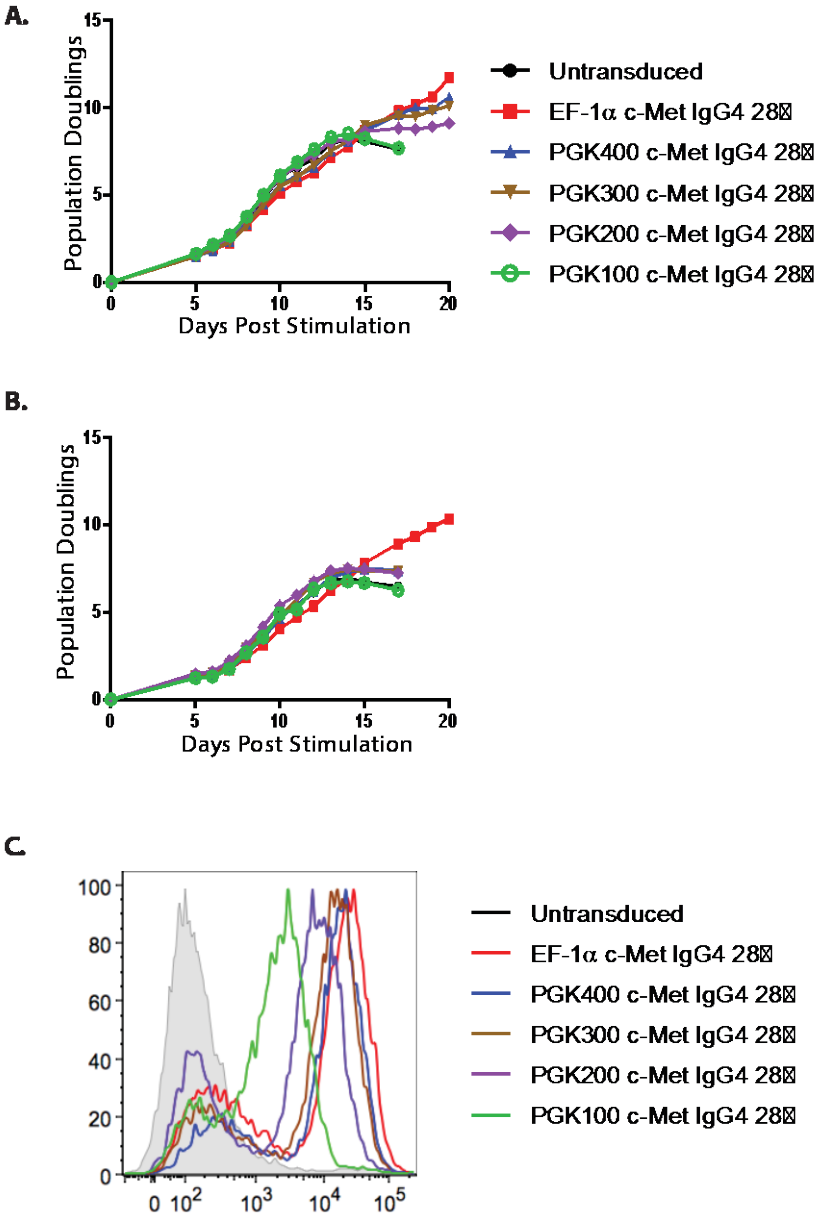
Supplemental Figure 7. Genome-wide microarray analysis of CAR T cells with constitutive proliferation. CD4+ T cells from 3 donors expressing continuous c-Met IgG4 or non-continuous CD19 CD8α CARs, or mock transduced cells were subjected to microarray analysis and hierarchical clustering from day 0 to day 24 of culture. Clustering was done using the euclidean distance of median normalized absolute log(2) gene expression intensities with average linkage. The plots are based on unbiased whole genome clustering. On day 11, CD19 CD8α CAR T cells and untransduced cells cluster more similarly to resting T cells, while day 11 and day 24 c-Met IgG4 CAR T cells remain activated and closely cluster.



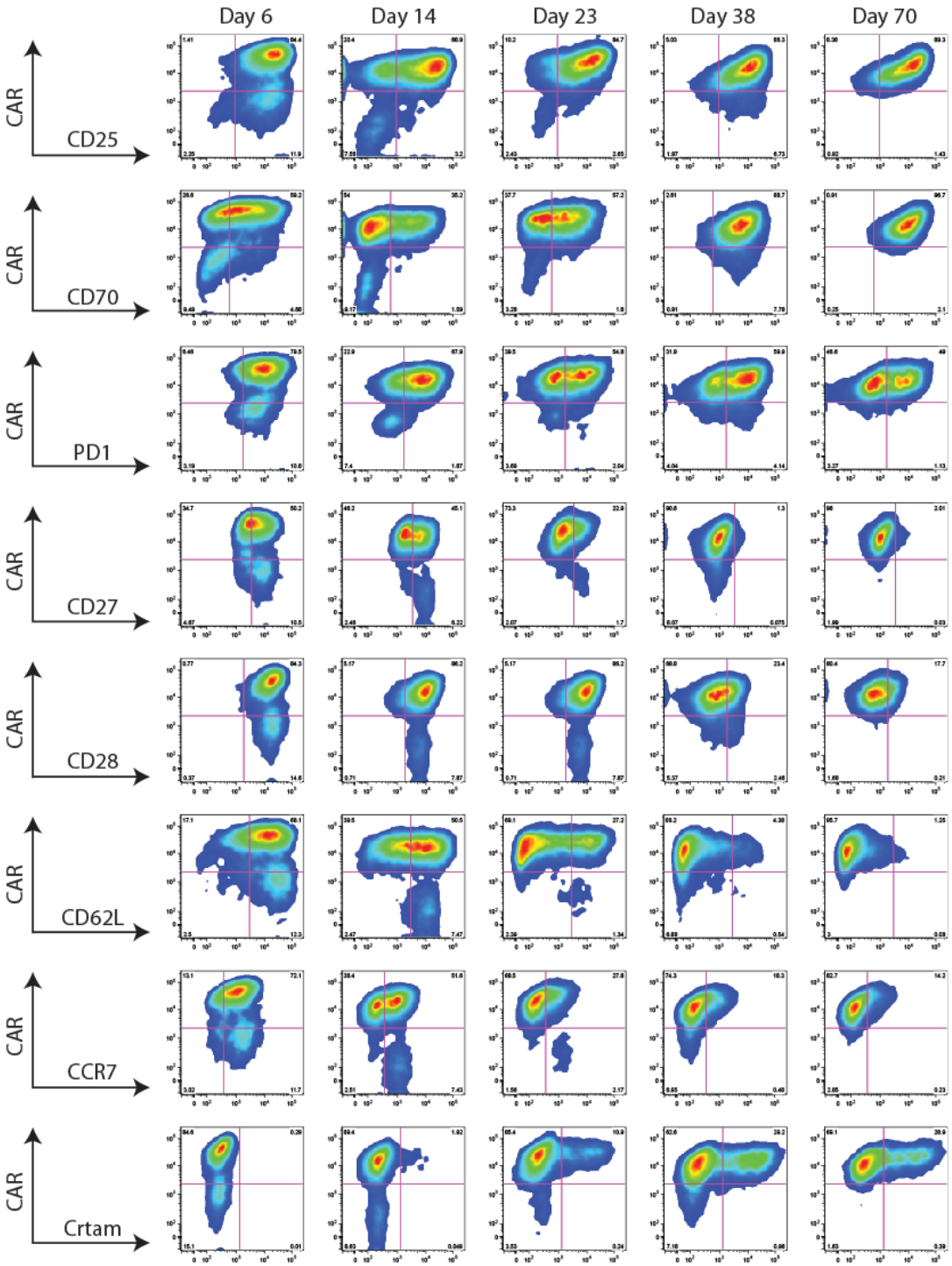
Supplemental Figure 8. Distinct gene expression signature of CAR T cells with constitutive proliferation. The gene expression signatures from the 3 donor T cell cultures on day 6 is compared to day 11 and day 24 cultures. CAR T cells on day 6 are similar to mock transduced T cells. In contrast, on day 11 and day 24 the continuous c-Met IgG4 cells display a unique RNA signature that differs from the fully activated day 6 phenotype.



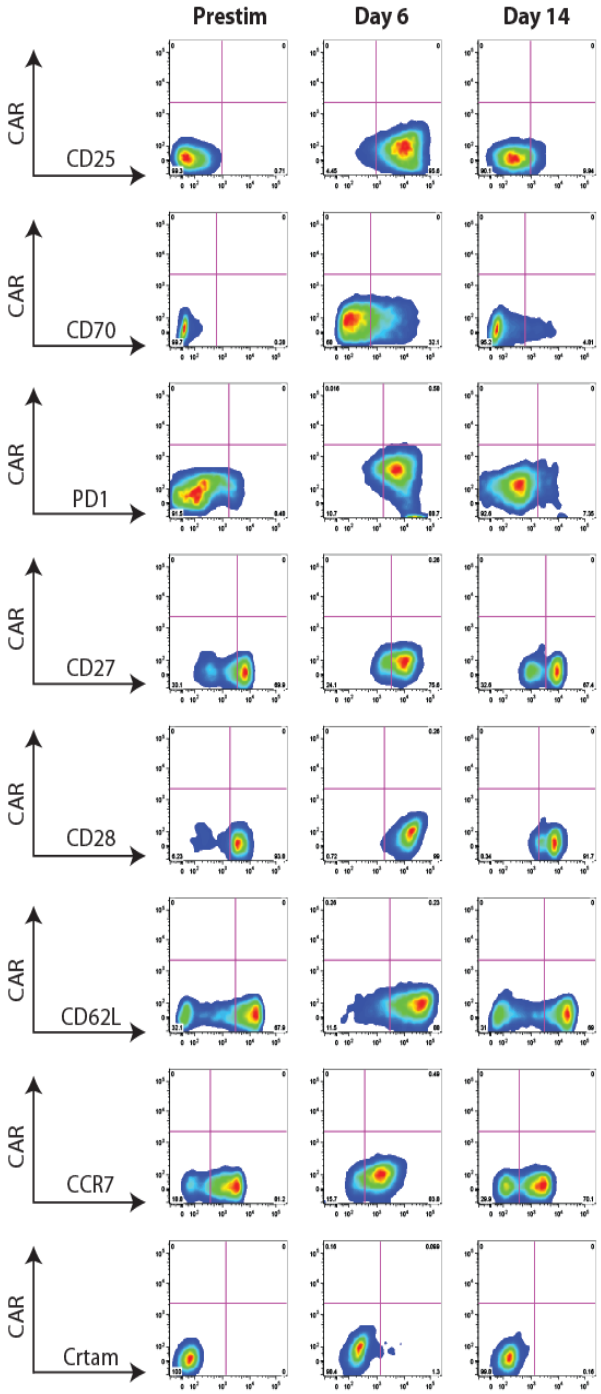
Supplemental Figure 9. CAR T cells with constitutive proliferation have ligand-independent NFAT activation. Jurkat T cells engineered to express GFP under the control of the NFAT promoter were transduced with lentivirus encoding CARs for continuous c-Met IgG4, SS1 IgG4, SS1 CD8α, and non-continuous CARs encoding CD19 IgG4, CD19 CD8α, and SS1 CD8aΔ tail. Cells were analyzed 3 days following transduction for GFP and CAR expression.



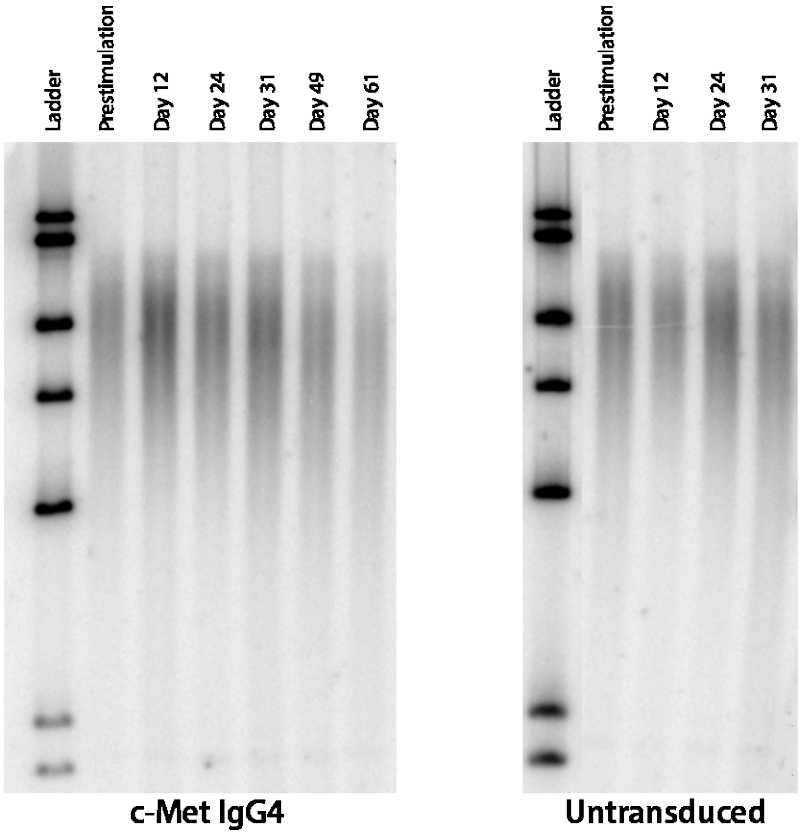
Supplemental Figure S10. Transgene expression levels are sufficient to convey the constitutive CAR growth phenotype. In vitro proliferation of human CD4+ (**A**) and CD8+ (**B**) T cells following 5 days of αCD3/CD28 coated magnetic bead stimulation and lentiviral transduction with c-Met IgG4 28ζ CARs expressed with the indicated promoter. (**C**) Comparison of the level of expression of CARs driven by each promoter is shown at 14 days post transduction.



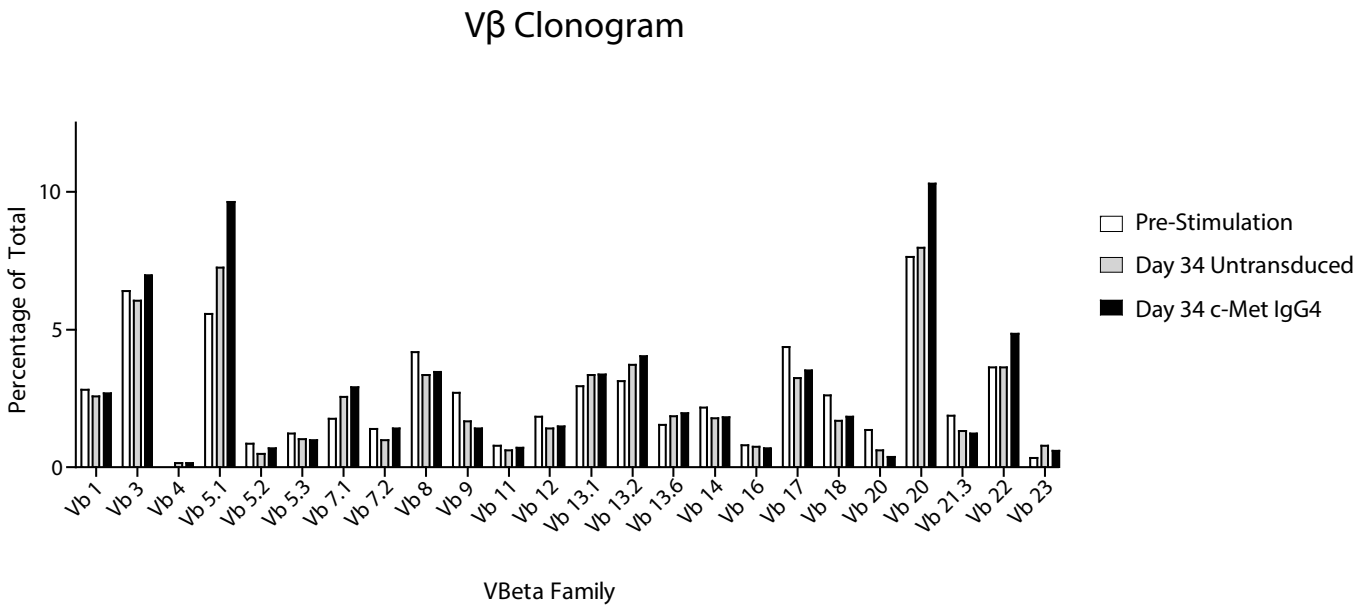
Supplemental Figure 11. Constitutive CAR T cell proliferation results in differentiation and evolution of a distinct cell surface phenotype. CD4 T cells were stimulated and transduced with the c-Met IgG4 CAR construct as previously described. Pre-stimulation cells were cryopreserved for later analysis. Cell samples were isolated at day 6, 14, 23, 38 and 70 and cryopreserved. Cells were thawed simultaneously and allowed to rest overnight without addition of cytokines. Cells were stained for CAR as well as CD25, CD70, PD-1, CD27, CD28, CD62L, CCR7 and Crtam as described in materials and methods.



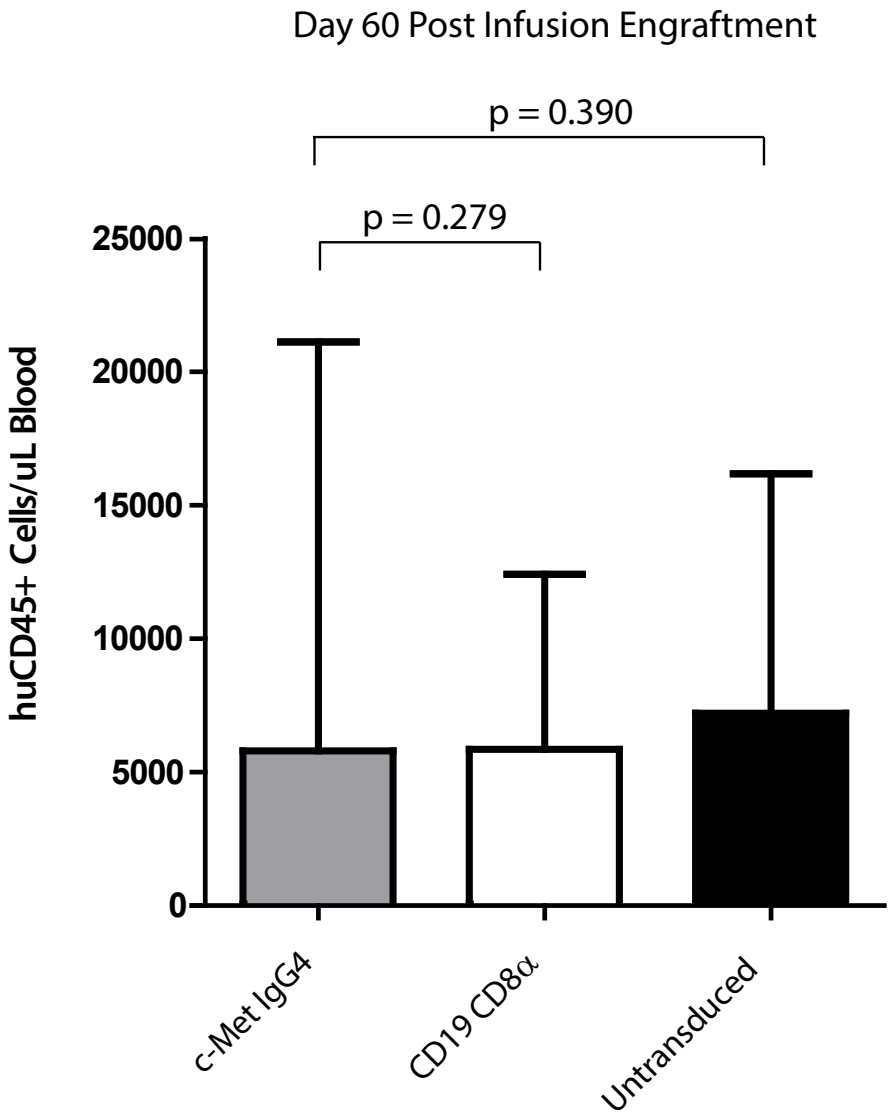
Supplemental Figure 12. Effects of stimulation and cell culture on differentiation of non-transduced T cells. Companion CD4 T cells were isolated via negative depletion and stimulated concurrently with the CAR T cells shown in supplemental figure 9. Pre-stimulation cells were cryopreserved for later analysis. Cell samples were isolated for analysis at day 6, 24 hours following bead removal, and on day 14. Cells were thawed simultaneously with the CAR T cells and rested overnight without additional of growth factors or cytokines. Cells were analyzed for CAR expression as well as CD25, CD70, PD-1, CD27, CD28, CD62L, CCR7 and Crtam.



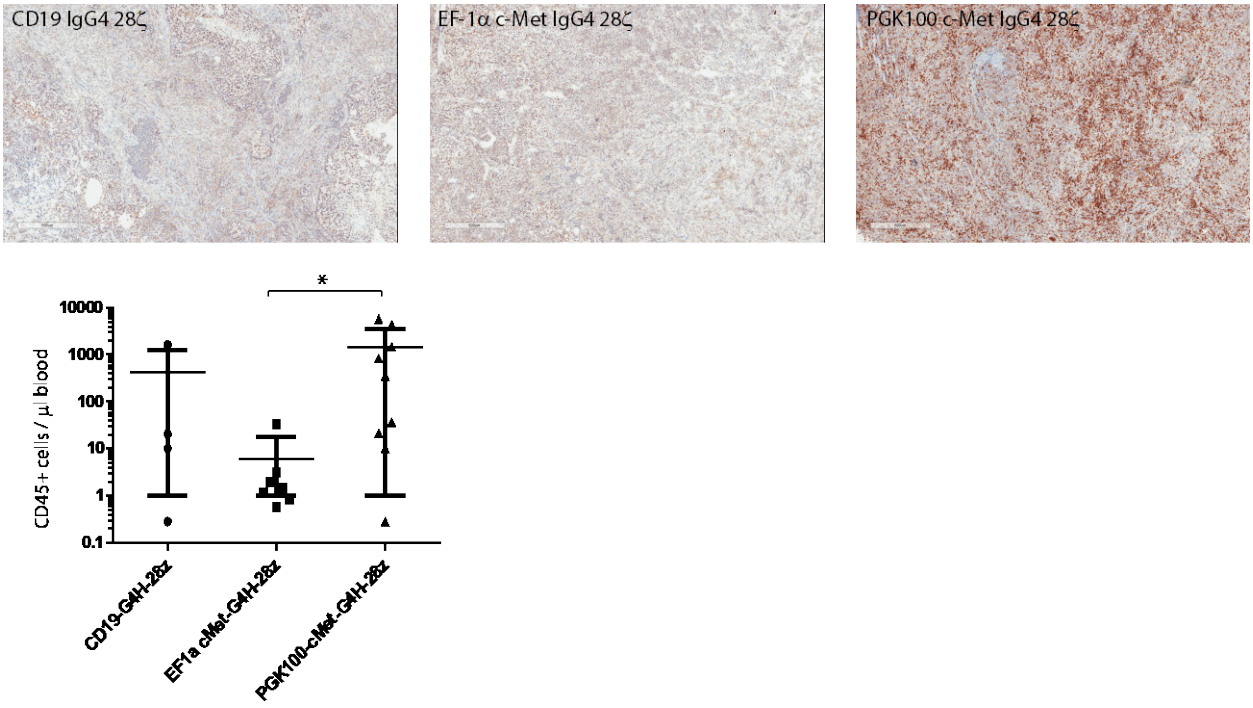
Supplemental Figure 13. Temporal patterns of telomere restriction fragment length (TRF) in continuous CAR T cells and mock transduced T cells. CD4 T cells transduced with the continuous c-Met IgG4 CAR or mock transduced cells were cultured for the indicated duration. DNA was isolated from the T cells and terminal telomeric restriction fragment length assessed by electrophoretic separation of HinfI/RsaI digested DNA followed by in-gel hybridization to a telomere repeat probe. The continuous CAR T cells proliferated for at least 61 days in culture while the mock transduced T cells ceased proliferation after 31 days. The ladder is 32P-labeled mixture of full-length and HindIII-digested lambda DNA.



Supplemental Figure 14. CAR T cells with a constitutive growth phenotype retain a diverse TCR Vβ repertoire. Human CD4+ T cells were isolated, stimulated with anti-CD3/CD28, transduced with c-Met IgG4 CAR, and maintained in culture without exogenous cytokines as described. Donor matched mock transduced cells were stimulated and expanded simultaneously as control, however these cultures required additional stimulations to maintain in culture. Cells were cryopreserved at days 0, 13 and 34 after which they were simultaneously thawed and TCR Vβ analysis was performed using the IOTest Beta Mark TCR V kit.



Supplemental Figure 15. Engraftment and proliferation of continuous CAR T cells in NSG mice. Human CD4 T cells (10^6) expressing the continuous c-Met IgG4 CAR, the non-continuous CD19 CD8α CAR (adjusted to 50% CAR positivity) or mock-transduced T cells were infused into NSG mice (n = 10 mice per group). Mice were analyzed 60 days following infusion by peripheral blood TruCounts to quantify huCD45+ cells per μL of mouse blood. Sample means were not different (two tailed Mann-Whitney p = 0.39); the bars denote S.D.



Supplemental Figure 16. PGK100 promoter results in antigen driven accumulation of CAR T cells in tissue but not blood. CD4+ and CD8+ T cells transduced to express CD19 IgG4 28ζ or c-Met IgG4 28ζ CAR under the influence of either EF1α promoter or PGK100 promoter were infused (two administrations, 16 x 10^6 cells in total) into mice bearing subcutaneous L55 tumors pre-established for 12 days. Top, Remaining subcutaneous tumor were sectioned and stained for CD3 to detect the presence of human T cells. Bottom, The absolute number of human CD45+ T cells was determined in the blood at the end of experiment; note log scale on Y-axis. * indicates $p < 0.05$. Two-tailed student T-test was used for statistical analysis.

4. Supplemental References

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