research; S.M. conducted research; J.S. designed research and conducted research; O.U.K. conducted research; S.G. conducted research and edited the manuscript; S.M. conducted research; A.D.P. conducted research and edited manuscript; S.A. designed research and provided essential protocols and reagents; L.J.N.C. designed research and provided essential protocols and reagents; J.P. conducted research; F.B.J. designed research and conducted research; C.M.P. designed research, conducted research and edited the manuscript; YZ designed research, conducted research and edited the manuscript; M.K. conducted research; M.C.M. conceptualized idea, supervised research, and edited manuscript; C.H.J. conceptualized idea, supervised research, and wrote manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST

M.J.F, Y.Z, J.S., M.K., M.C.M and C.H.J. have intellectual property rights, including patents, to some of the cell culture technologies and CARs described in the manuscript. A.P, M.C.M and C.H.J have sponsored research grants from Novartis. L.J.N.C received honoraria from Speakers Bureau from Miltenyi, and has ownership interests, including patents, with Targazyme, and is a consultant with Ferring Pharmaceuticals, Janssen Pharmaceuticals and Cellectis. All other authors declare no competing financial interests. Conflicts of interest are managed in accordance with University of Pennsylvania policy and oversight.

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FIGURE LEGENDS

Figure 1. Induction of constitutive, ligand-independent CAR T-cell proliferation.
A) *In vitro* proliferation of human $CD4^+$ T cells following 5 days of αCD3/CD28-coated magnetic bead stimulation and lentiviral transduction with the indicated CAR constructs (left panel). No cytokines were added to culture media at any point during expansion. The CAR T cells with constitutive proliferation also maintain a larger mean cell volume (right panel). Results are representative of n>10 normal human donors. **B and C**) $CD4^+$ and $CD8^+$ T cells were stimulated as in (A), with or without exogenous IL2. *In vitro* proliferation of human $CD4^+$ (**D**) T cells following lentiviral transduction with the indicated CAR constructs. No cytokines were added to culture media at any point during expansion for $CD4^+$ T cells. Results are representative of n>4 normal human donors. **E)** $CD4^+$ T cells from 3 healthy donors were isolated, stimulated and transduced with lentivirus encoding the c-Met IgG4, CD19 CD8-α, and CART19 CAR constructs or mock transduced, and cultured with addition of fresh media and no exogenous cytokines. Error bars denote standard deviation. The design of the various CAR constructs is shown in Supplemental Figure S1.

Figure 2. CAR T cells with continuous T-cell proliferation have constitutive cytokine secretion.
Serial measurements of cytokine production by various CAR constructs following αCD3/CD28 stimulation and expansion. At each noted time point c-Met IgG4, CD19 IgG4 CAR transduced, and untransduced $CD4^+$ T cells were collected from culture, washed and re-plated at $1x10^6$/mL. Cells were kept in culture for 24 hrs at which time supernatant from each culture was collected. Supernatants were analyzed via luminex assay and values plotted as log(10) fold-change from the pre-stimulated cells (baseline). Baseline values (pg/ml) for each analyte were: IFNγ: 5 pg/mL; TNFα: 2 pg/mL; IL2: 1 pg/mL; GM-CSF: 15.25 pg/mL; IL13: 1 pg/mL; IL10: 1 pg/mL. The design of the CAR constructs is shown in Supplemental Figure S1.

Figure 3. CARs with a constitutive growth phenotype display a unique gene signature. Cytokines, perforin and granzyme expression. Microarray analysis comparing cytokine expression of c-Met IgG4 (green), CD19 CD8-α (red), CART19 (blue) CARs and untransduced (orange) T cells at baseline and on days 6, 22 and 24 of culture; only the c-Met IgG4 culture was analyzed on day 24 because the other cultures were terminated due to cell death. No exogenous cytokines were added to the culture media. Normalized absolute log_2 gene expression intensities are plotted for IFNγ, TNFα, IL17A, IL2, IL3, IL4,

GM-CSF, IL10, IL13, Granzyme B and Perforin, The design of the CAR constructs is shown in Supplemental Figure S1.

Figure 4. Constitutive activation of AKT, NF-kB and MAPK signaling pathways is associated with the CAR T-cell proliferative phenotype. A) Representative FACS histograms displaying enrichment of c-Met IgG4 CAR$^+$ T cells during culture from day 10 to day 30 of culture. **B)** PhosFlow plots of CD4$^+$ T cells stimulated and transduced with the c-Met IgG4 constitutive or CD19 CD8α non-constitutive CARs as previously described. On days 6, 10 and 25 cells were fixed, permeabilized and stained using PE anti-Erk1/2 (pT202/pY204), PE anti-Akt (pS473), PE anti-NF-kB p65 (pS529) and PE anti-S6 (pS235/pS236); the CD19 CD8α CAR culture did not continue to proliferate to day 25, and therefore is only analyzed on days 6 and 10. Positive controls were samples from each condition stimulated for 10 min using PMA/Ionomycin prior to fixation, while negative controls cells were fully stimulated T cells stained using PE-conjugated IgG2b κ isotype control. The design of the CAR constructs is shown in Supplemental Figure S1.

Figure 5. Heat map showing relative intensities of the differentially expressed genes in CD4$^+$ T cells expressing continuous CARs or non-continuous CARs. The differentially expressed genes with a 5-fold cutoff in CD4$^+$ cells from 3 healthy donors are shown for c-Met IgG4 CAR and CD19 CD8α CAR on day 11. The expression level of each gene is represented by the number of standard deviations above (red) or below (blue) the average value for that gene across all samples. The list of the differentially expressed genes is shown in Supplemental Tables S2 and S3. The design of the CAR constructs is shown in Supplemental Figure S1.

Figure 6 A,B and C. Transgene expression levels are sufficient to convey the constitutive CAR growth phenotype. *In vitro* proliferation of human CD4$^+$ T cells following 5 days of anti-CD3 plus anti-CD28 stimulation and lentiviral transduction with c-MET-expressing CARs under the control of the indicated promoter. CMV(1) and CMV (2) represent replications of lentiviral vector production in the same human donor. **A)** Population doublings were determined for both CMV and EF-1α driven c-MET CAR cells. After ~12 days in culture, CMV-c-MET CAR cells were unable to sustain proliferation and died, while EF-1α c-MET CAR T cells continue to proliferate. **B)** Mean cell volume (MCV) was also determined. The CMV-c-MET CAR T cells decreased in cell size after 10 days, indicative of the cells resting down. **C)** Comparison of the level of expression between CARs expressed with the CMV and EF-1α promoters is shown at day 6 post-transduction. The mean fluorescence intensity is indicated. The design of the CAR constructs is shown in Supplemental Figure S1.

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Figure 7A-C. *In vivo* efficacy of c-Met IgG4 28ζ CAR T cells. $CD4^+$ and $CD8^+$ T cells transduced to express CD19 IgG4 28ζ or c-Met IgG4 28ζ CAR under the influence of either EF-1α promoter or PGK100 promoter were infused (two administrations, 16 x 10^6 cells in total) into mice (for no T cells n=2; for the rest n=8 per group) bearing intraperitoneal SKOV3 tumors pre-established for 16 days. (**A**) Bioluminescence signal was acquired every week as a surrogate for tumor growth. $p < 0.01$ EF-1α vs PGK100 group. (**B**) Kaplan-Meier analysis. * indicates $p < 0.05$, EF-1α vs PGK100, log-rank (Mantel-Cox) test was used for statistical analysis. (**C**) The absolute number of human $CD45^+$ T cells was determined in the blood on days 37 (left panel) and 73 (right panel), respectively. Only 2 mice survived in the EF-1α c-Met IgG4 28ζ CAR group on d73. * indicates $p < 0.05$. Two-tailed student T-test was used for statistical analysis. The design of the CAR constructs is shown in Supplemental Figure S1.

Fig. 1

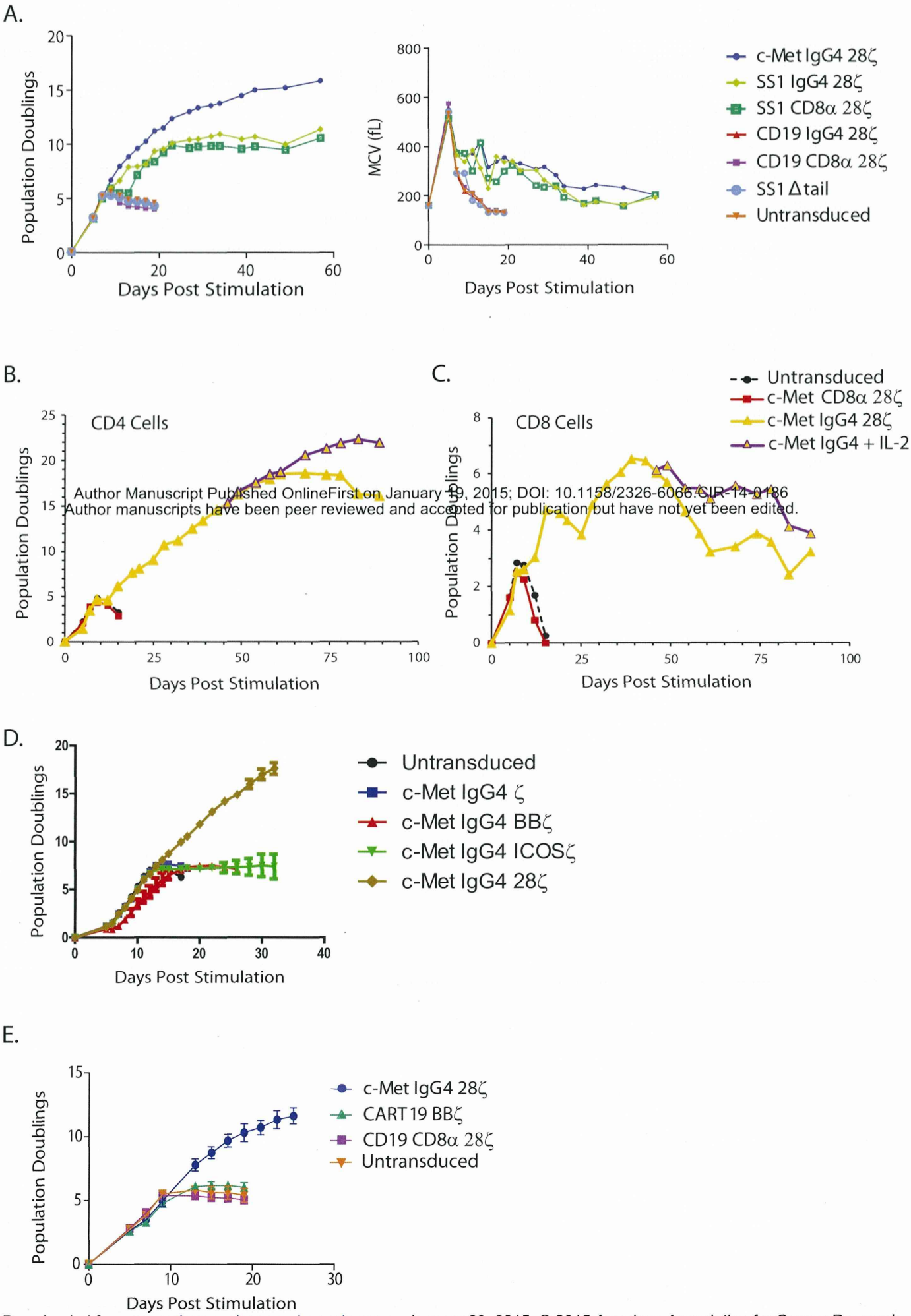

Fig 2