SUPPLEMENTARY INFORMATION

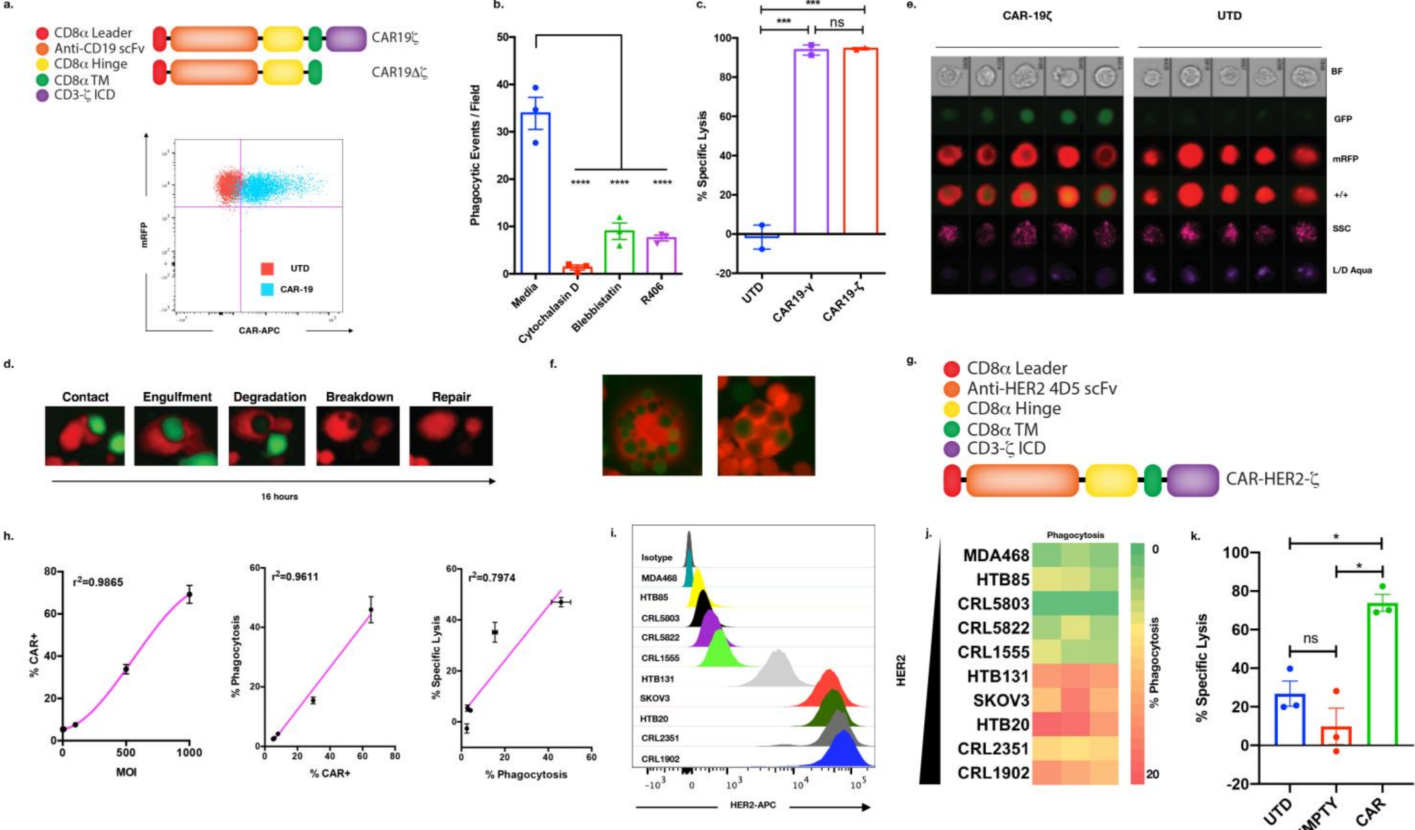
https://doi.org/10.1038/s41587-020-0462-y

In the format provided by the authors and unedited.

Human chimeric antigen receptor macrophages for cancer immunotherapy

Michael Klichinsky[1,2,3], Marco Ruella[1,4], Olga Shestova[1], Xueqing Maggie Lu[1,5], Andrew Best[1,3], Martha Zeeman[3], Maggie Schmierer[3], Konrad Gabrusiewicz[3], Nicholas R. Anderson[3], Nicholas E. Petty[1], Katherine D. Cummins[1], Feng Shen[1], Xinhe Shan[1], Kimberly Veliz[1], Kristin Blouch[1], Yumi Yashiro-Ohtani[3], Saad S. Kenderian[1,8], Miriam Y. Kim[1,9], Roddy S. O'Connor[1], Stephen R. Wallace[1], Miroslaw S. Kozlowski[1], Dylan M. Marchione[2,6], Maksim Shestov[1], Benjamin A. Garcia[6], Carl H. June[1,2,7] and Saar Gill[1,2,4] ✉

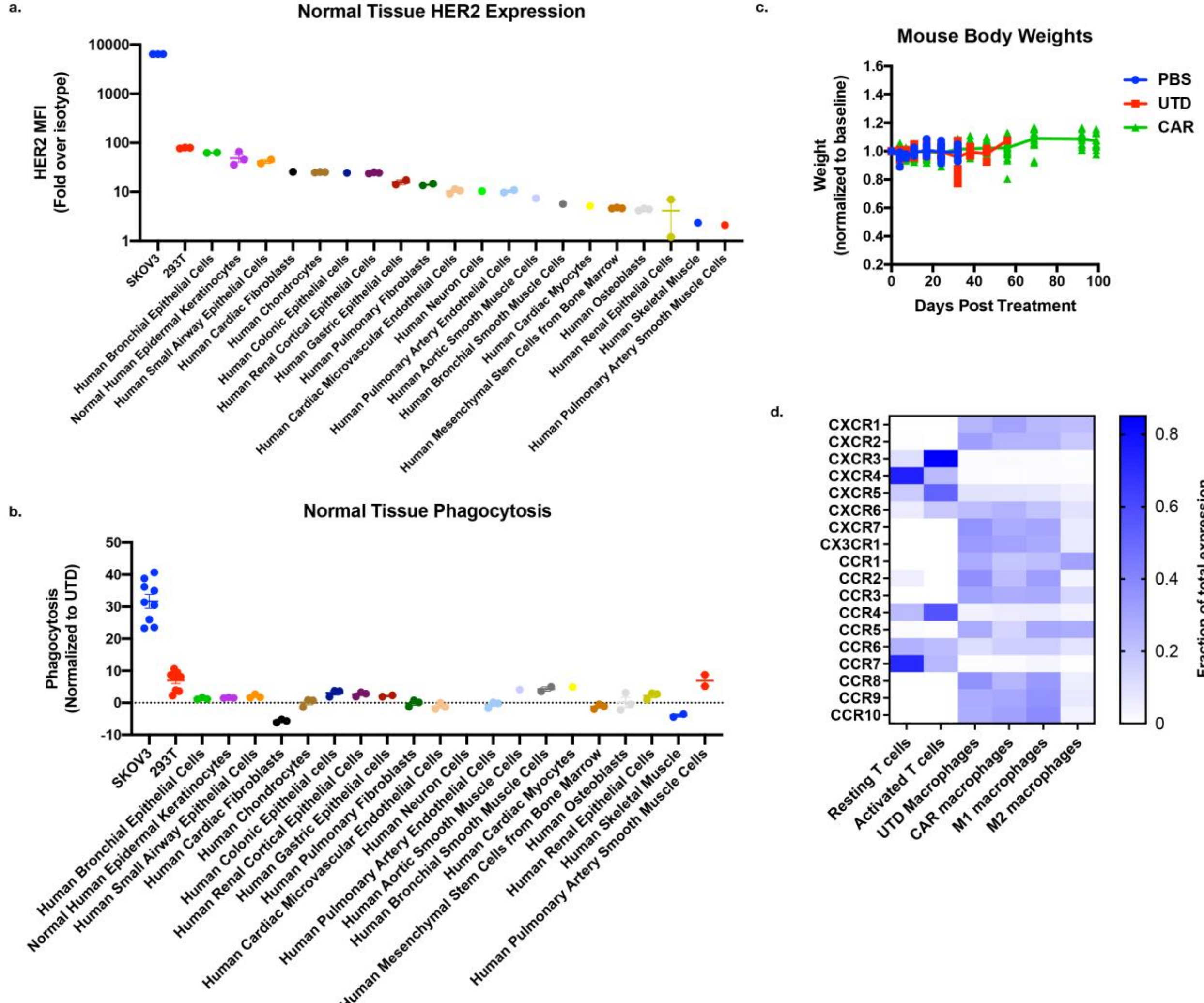
[1]Center for Cellular Immunotherapies, University of Pennsylvania School of Medicine, Philadelphia, PA, USA. [2]Department of Systems Pharmacology and Translational Therapeutics, University of Pennsylvania School of Medicine, Philadelphia, PA, USA. [3]Carisma Therapeutics, Philadelphia, PA, USA. [4]Division of Hematology-Oncology, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA, USA. [5]Institute for Biomedical Informatics, University of Pennsylvania, Philadelphia, PA, USA. [6]Department of Biochemistry and Biophysics, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA. [7]Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA, USA. [8]Present address: Division of Hematology, Mayo Clinic, Rochester, MN, USA. [9]Present address: Department of Medicine, Oncology Division, Washington University School of Medicine, St. Louis, MO, USA. ✉e-mail: saar.gill@pennmedicine.upenn.edu



Supplementary Figure 1

Mechanistic characterization of CAR macrophage phagocytosis.

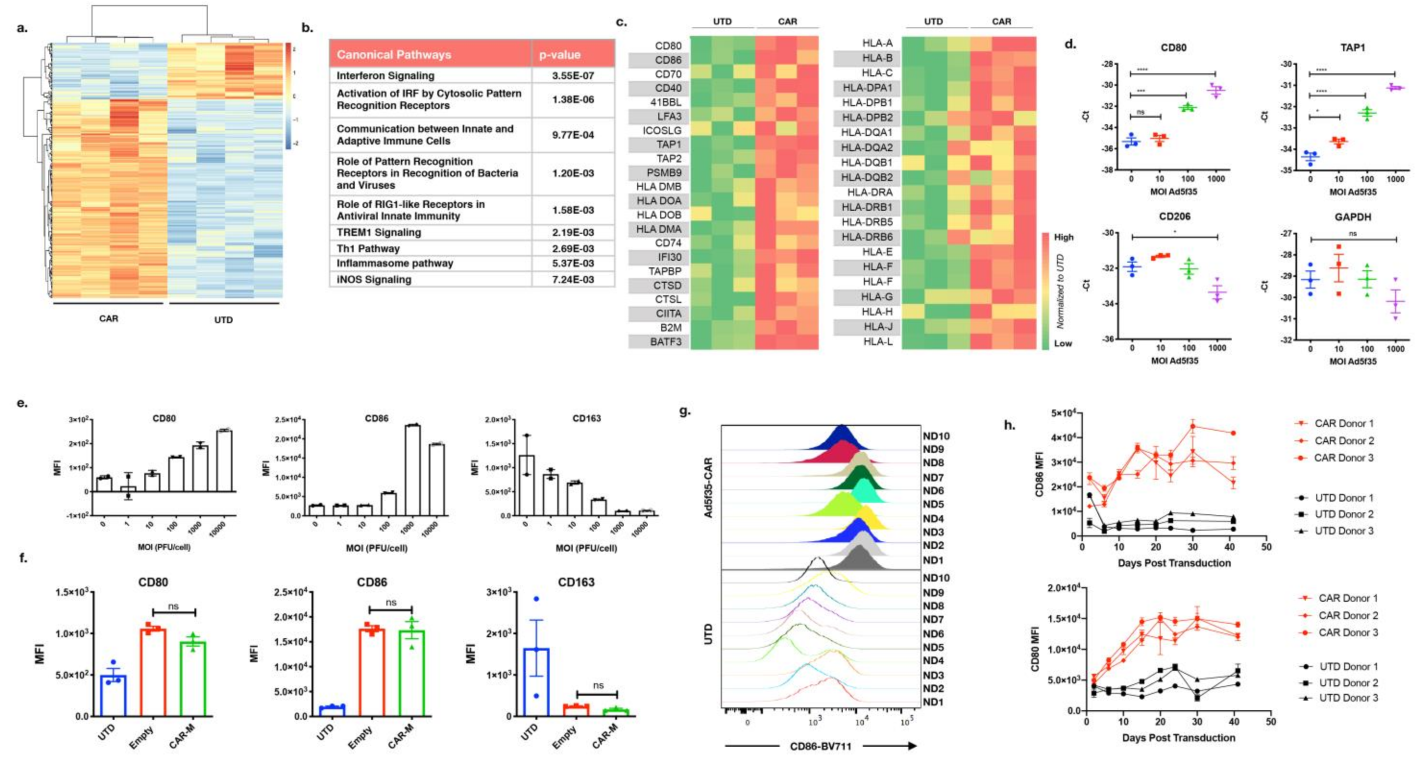
a, Constructs utilized in lentiviral vectors to express CAR-19 variants in THP-1 cells (left). Representative FACS plot of CAR19 expression (post-sort) in mRFP+ THP-1 cells (right). FACS plot is representative of at least 3 experiments. **b**, CAR19ζ+ THP-1 macrophages were pre-treated with media, or the phagocytosis inhibitors cytochalasin-D, blebbistatin, or R406 prior to the phagocytosis assay. Data represent the mean +/- SEM of (n) = 3 technical replicates. Statistical significance was calculated via ANOVA with multiple comparisons. **c**, Phagocytosis of CD19+ K562 target cells by CAR19ζ + or CAR19γ + (a CAR based on the Fc gamma chain) THP-1 macrophages. Data represent the mean +/- standard error (SEM) of (n)=3 technical replicates. Statistical significance was calculated via one-way ANOVA with multiple comparisons. d, Representative in vitro microscopy demonstrating steps in the CAR macrophage phagocytic process. A single macrophage was tracked over 16 hours. Images are representative of at least 3 experiments. e, Imaging cytometry of UTD or CAR19ζ mRFP+ THP-1 macrophages after co-culture with GFP+ CD19+ K562 target cells. Experiment was performed once. f, Representative image of poly-phagocytic CAR19ζ THP-1 macrophages from 4-hour co-culture at a 1:1 effector to target ratio. Experiment was performed at least 3 times. g, Diagram of the anti-HER2 CAR construct engineered into the Ad5f35 vector under the control of a CMV promoter. h, Human macrophages were transduced with CAR-HER2-ζ Ad5f35 at MOIs of 0, 100, 500, or 1000 PFU. CAR expression correlated with MOI (left), in vitro phagocytosis against SKOV3 (middle), and in vitro cytotoxicity against SKOV3 at 48 hours (right). Data are represented as mean +/- SEM of (n)=3 technical replicates. Correlation was determined via linear regression and Pearson correlation. i-j, A panel of 10 human cancer cell lines were tested for surface HER2 expression (isotype and MDA-468 are negative controls). These cell lines were used as targets for CAR-HER2-ζ macrophage phagocytosis. Percent phagocytosis is shown as a heatmap, with each column representing a different donor. Cell lines are ordered by HER2 expression (low-to-high). Experiment was performed twice. k, Luciferase based killing assay of SKOV3 by UTD, empty-vector Ad5f35 transduced (Empty), or anti-HER2 CAR primary human macrophages (CAR) at 48 hours. Data represent the mean +/- SEM of (n)=3 technical replicates; statistical significance was calculated with multiple two-sided t-tests. For all panels: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



Supplementary Figure 2

Anti-HER2 CAR macrophages do not phagocytose normal tissue.

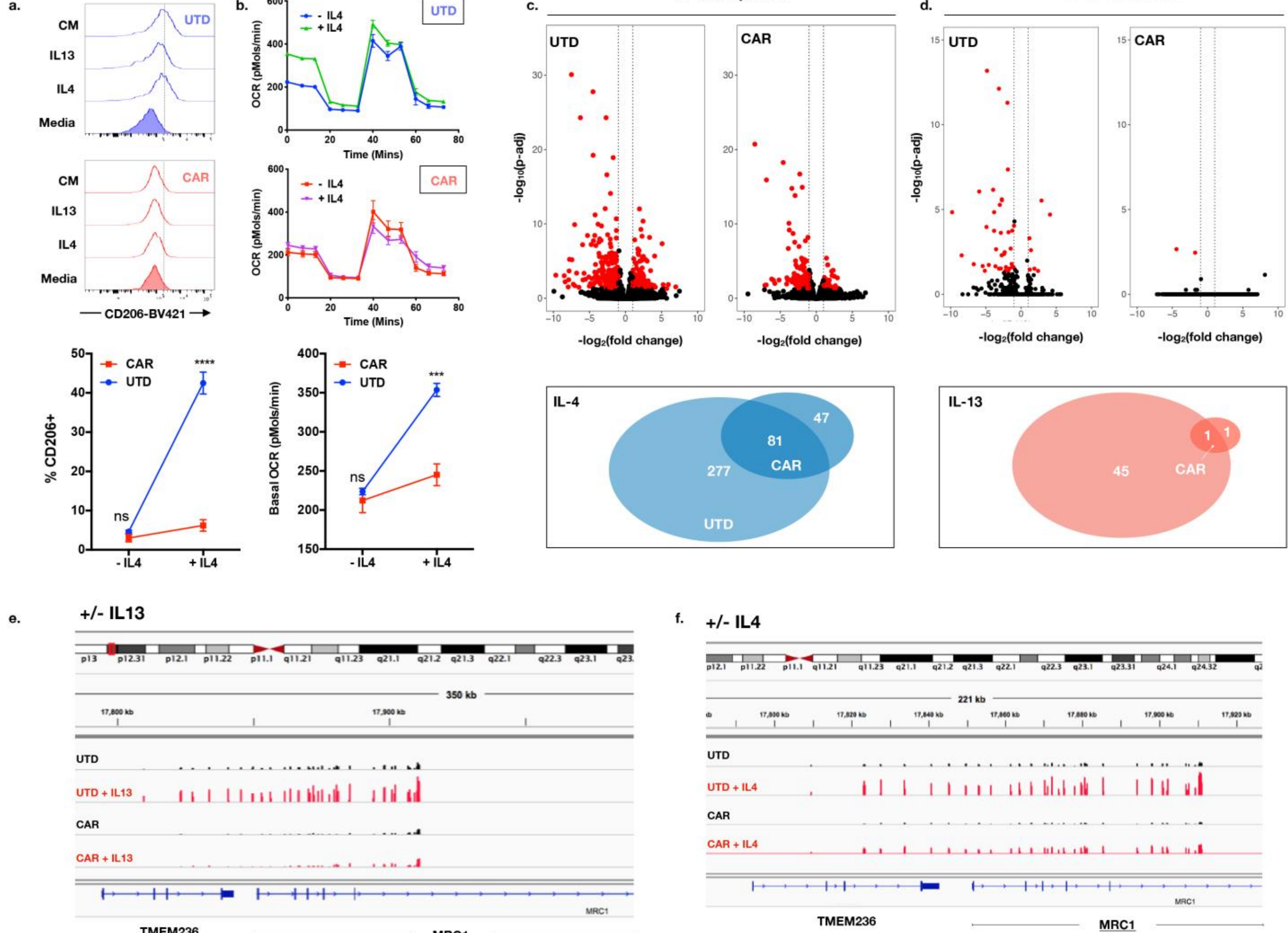
a, HER2 mean fluorescence intensity (fold over isotype control) on SKOV3 (HER2 high control), 293T (HER2 low control), or a panel of normal human tissues. The data represents mean +/- SEM of (n)=2-3 technical replicates per tissue type. **b**, Phagocytosis of CFSE labeled SKOV3, 293T, or normal human cells by anti-HER2 CAR macrophages (normalized to UTD macrophages to correct for background fluctuations for each target cell type). Target cells are ordered from HER2 high to low (left-to-right) on the x-axis. The data represents mean +/- SEM of (n)=1-3 technical replicates per tissue type. **c**, Mouse body weights after IP injection with PBS, UTD, or CAR-HER2 macrophages (normalized to day 0 for each mouse). Data represents the mean for (n)=12 (PBS), 9 (UTD), and 23 (CAR-M) mice. **d**, FACS based characterization of chemokine receptor expression of human resting T cells, CD3/CD28 antibody activated T cells, UTD macrophages, CAR macrophages, classically activated M1 macrophages (IFNγ/LPS), or alternatively activated M2 macrophages (IL-4). The relative expression for each chemokine receptor is plotted as a heatmap. Data represent averages from at least 3 donors. For all panels: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



Supplementary Figure 3

Ad5f35 transduction leads to a pro-inflammatory (M1) macrophage phenotype.

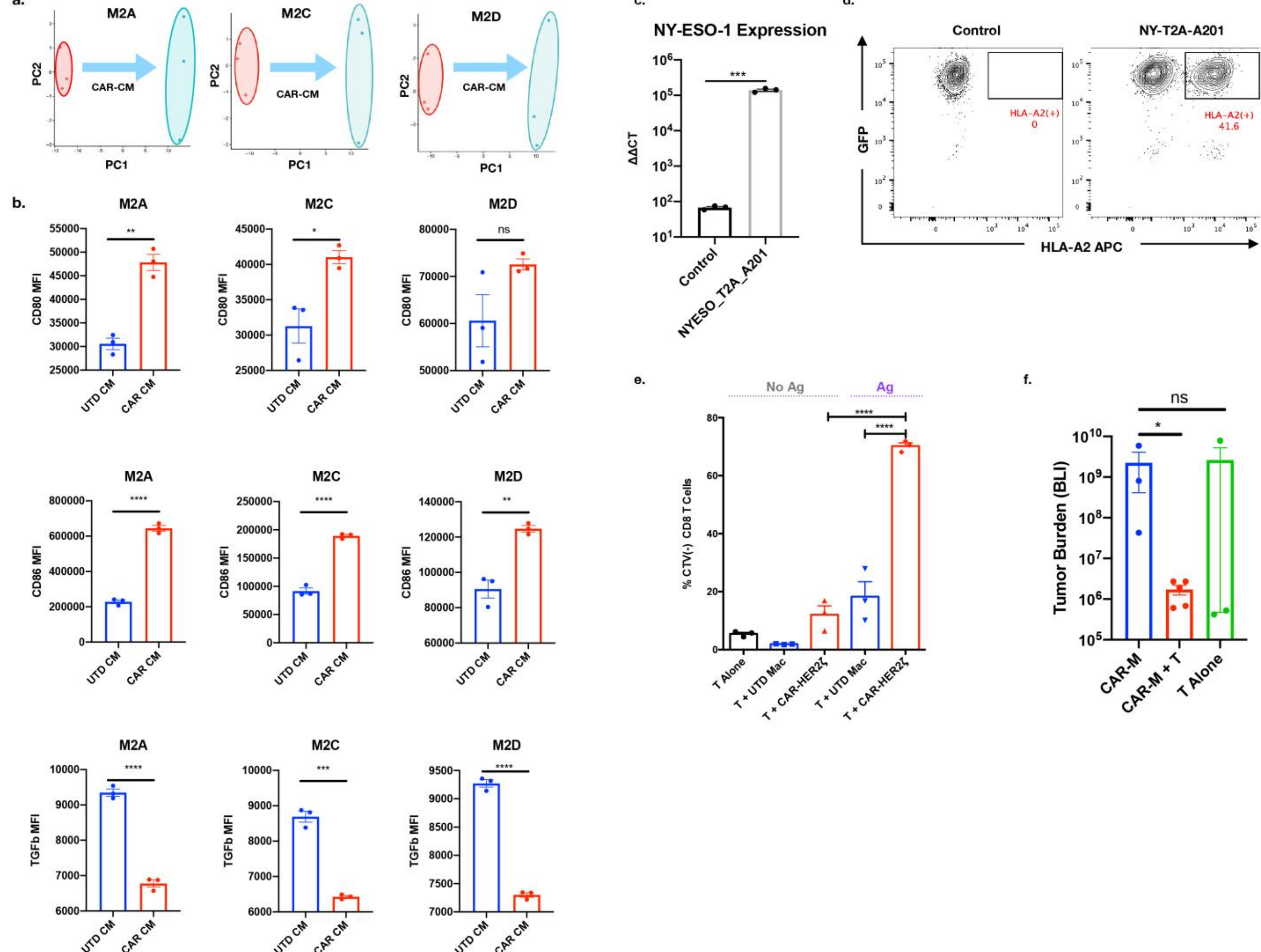
a, Hierarchical clustering of differentially expressed genes from UTD or Ad5f35-CAR-HER2-ζ transduced human macrophages from (n) = 4 matched donors, 48 hours post transduction. The heatmap shows $\log_2$ fold-change in gene expression relative to UTD. **b**, Table of Ad5f35 induced canonical pathways in human macrophages, derived from (n) = 4 matched donors. Statistical analysis was performed using Fisher's exact test. **c**, Heatmap of differentially expressed co-stimulatory ligands, antigen processing genes, and MHC-I/MHC-II genes between UTD and Ad5f35 transduced CAR macrophages via RNA-seq. **d**, Confirmation of select RNA-seq results in (3c) via RT-qPCR. Data represent the mean +/- SEM of (n)=3 technical replicates. Statistical analysis was performed using one-way ANOVA with multiple comparisons. **e**, Mean fluorescence intensity of human M1 markers CD80 and CD86 and M2 marker CD163 in response to transduction with increasing MOIs of Ad5f35-CAR by FACS. Data is represented as mean +/- SEM of (n)=2 technical replicates. Experiment was repeated at least 3 times. **f**, Surface expression of human M1 markers (CD80 and CD86) and M2 marker CD163 after transduction with equivalent MOIs of control empty-vector Ad5f35 or Ad5f35-CAR. Data is represented as mean +/- SEM of (n)=2 technical replicates. Experiment was repeated at least 3 times. **g**, Surface expression of M1 marker CD86 on control UTD or Ad5f35-CAR transduced macrophages from (n)=10 human matched-donors. **h**, Persistence of M1 marker expression (CD80 and CD86) on primary human UTD or CAR macrophages from (n)=3 human donors over the course of 40 days of in vitro culture. For all panels: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



Supplementary Figure 4

Ad5f35 transduced CAR macrophages are resistant to M2 inducing cytokines.

a, Upregulation of CD206 in response to M2-challenge in UTD or CAR macrophages (representative histograms; top panel, %CD206(+) in response to IL-4; bottom panel). Data is shown as mean +/- SEM from (n)=3 technical replicates; statistical significance was calculated with a two-sided t-test and the experiment was performed two times. **b**, The change in oxygen consumption rate (OCR) upon treatment with IL-4 in UTD or CAR macrophages (representative OCR diagrams, top panel; mean basal OCR; bottom panel). Data is shown as mean +/- SEM from (n)=3 technical replicates; statistical significance was calculated with a two-sided t-test; experiment was performed one time. **c-d**, Volcano plot of IL4 (4c) or IL13 (4d) response genes in UTD or CAR macrophages from (n)=3 human donors. Red indicates p-adj <0.05 and $\log_2$ fold change >1 or <-1. Statistical significance was calculated using the Wald test for DESeq2 data. Venn diagrams show the number of M2-cytokine induced genes in UTD, CAR, or both macrophage types. **e-f**, RNA-seq reads mapped to the MRC1 gene locus, from UTD or CAR macrophages stimulated +/- IL4 (4e) or +/- IL13 (4f). For all panels: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



Supplementary Figure 5

Ad5f35 transduced CAR-M convert M2 macrophages toward M1 and activate T cells.

a, Principle component analysis plots demonstrating the phenotypic shift of M2A, M2C, or M2D macrophages after treatment with control UTD macrophage conditioned media (red) or CAR macrophage conditioned media (blue). Data was derived from (n)=3 technical replicates. **b**, Evaluation of surface expression of M1 markers CD80 and CD86 or intracellular expression of the immunosuppressive cytokine TGF-β on M2A, M2C, or M2D macrophages after treatment with UTD or CAR CM for 48 hours. Data represent mean +/- SEM from (n)=3 technical replicates; statistical significance was calculated with a two-tailed t-test. **c**, Real-time PCR confirmation of expression of NY-ESO-1 after transduction of SKOV3 cells with a lentivirus co-expressing NYESO and HLA-A201. Data represents mean +/- SEM of (n)=3 technical replicates. Experiment was performed twice. **d,** Representative FACS plot confirming HLA-A201 expression after transduction of SKOV3 cells with a lentivirus co-expressing NYESO and HLA-A201. Experiment was performed twice with similar results. **e**, Control or NY-ESO-1 expressing macrophages (No Ag and Ag, respectively), with or without Ad5f35-CAR were co-cultured with CTV-labeled anti-NY-ESO-1 T cells. Proliferation of anti-NY-ESO-1 TCR+ CD8+ T cells is shown as mean +/- SEM of (n)=3 technical replicates; statistical significance was determined using ANOVA with multiple comparisons. **f**, Tumor burden of NSGS mice with metastatic SKOV3 that were treated IV with CAR-M alone, CAR-M + donor derived non-engineered/non-expanded T cells, or T cells alone, 63 days post treatment. Data represents mean +/- SEM from (n)=3-5 mice per group, as indicated in the figure; experiment was performed once; statistical analysis was calculated with a Mann-Whitney U test. For all panels: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.