Supplementary I:

Materials and Methods

Cell lines and oncolytic virus. HEK293, SKOV3, CT26, Vero, BHK, TALL-104 cells were obtained from ATCC. CT26-EGFR cells were established from CT26 cells by stably transducing the cells with a lentiviral vector that contains EGFR extracellular and transmembrane domains without the intracellular sequence[1]. All cells were maintained in DMEM medium with 10% FBS, except TALL-104 cells, which were cultured in RPMI medium supplemented with HI-FBS and IL-2 at 100 ng/mL concentration at 37ºC with 10% CO2.

Synco-2D is an HSV-1–based oncolytic virus. Its construction has been described in our previous publications[2]. Briefly, it has both copies of the *ICP34.5* gene deleted. Additionally, it contains two membrane fusion mechanisms - the syn phenotype through mutagenesis and insertion of the truncated form of the gibbon ape leukemia virus envelope fusogenic membrane glycoprotein (GALV.fus) into the virus genome[2].

Plasmid construction. For building the BiCEP, the coding sequence for OMCP and the mutated form of EGFα (m123), together with a glycine/serine linker and a Myc tag was synthesized by GenScript (Piscataway NJ). TriCEP coding sequence was similarly synthesized, except that the coding sequence for a mutated form of IL-2 was added to the 5' end. Both synthesized sequences were cloned into pcDNA3.1 plasmid to generate pcDNA3.1-BiCEP and pcDNA3.1-TriCEP, respectively.

Amplicon Plasmid cloning and Amplicon production. The pcDNA3.1 plasmids containing the BiCEP and TriCEP sequences were constructed as mentioned above. For constructing amplicon plasmids containing these two chimeric engagers, the key components of an HSV amplicon, the Ori and the Pac signals, together with the EGFP coding sequence, were cut from pW7-EGFP, which is an amplicon that our lab had constructed and used in many of our previous studies[3]. The cut-out fragment containing the amplicon components was then cloned into pcDNA3.1-BiCEP and pcDNA3.1-TriCEP, to generate Amplicon-BiCEP and Amplicon-TriCEP, respectively. For packaging the amplicon plasmids into HSV viral particles, Amplicon-BiCEP and Amplicon-TriCEP were transfected to BHK cells with Fugene HD (Promega, Madison, WI). pW7-GFP amplicon vector was included as a control. The transfected cells were infected with Synco-2D at 0.1 pfu/cell 24 h later. The cells were harvested when full cytopathic effect (CPE) was detected. Synco-2D and the packaged amplicon were released by three cycles of freeze-thaw, followed by centrifugation. The generated stocks were labeled as Synco-2D-GFP, Synco-2D-BiCEP, and Synco-2D-TriCEP, respectively, and stored at -80C until use.

In vitro detection of transgene expression in mammalian cells

For determining the transgene expression from either the amplicon plasmids or from the packaged amplicons, HEK293 cells were transfected with pW7-GFP, Amplicon-BiCEP and Amplicon-TriCEP, and BHK cells were infected with the corresponding packaged amplicons. The supernatants were collected 48 and 72 h later. The collected supernatants were either used directly or concentrated using 10,000 MWCO Millipore spin-columns and stored at -80C before they were used for Western blot detection or other quantitative assays.

Binding assays by flow cytometry analysis. The binding of OMCP to NKG2D was determined by incubating TALL-104 cells with the supernatants collected from HEK293 cells transfected with the amplicon plasmids or BHK cells infected with the packaged amplicon as described above. After one h incubation at room temperature with the fusion proteins containing supernatants, the cells were stained for NKG2D (with APC conjugated anti-human CD314 (NKG2D) antibody (BioLegend, San Diego, CA) and Myc-tag with PE-conjugated Myc-tag mouse mAb (Cell Signaling Technology, Danvers, MA) for 30 mins at 4ºC. Post staining, the cells were washed three times with 2%FBS containing PBS to remove any excess antibody. After the final wash, the cells were resuspended in flow staining buffer and analyzed immediately for the double staining for OMCP to NKG2D (determined by detection of $NKG2D^+/Myc^+$ double-positive cells) by flow cytometry. Similarly, binding of EGFα to EGFR on SKOV3 cells and on CT26-EGFR cells is determined by incubating the cells with the supernatants collected from HEK293 cells transfected with the amplicon plasmids or BHK cells infected with the packaged amplicon respectively, for 30 mins at RT. The cells were then stained for EGFR with Brilliant Violet-421 conjugated anti-human EGFR antibody (BioLegend, San Diego, CA) and Myc tag with PE-conjugated Myc-tag mouse mAb (Cell Signaling Technology, Danvers, MA). The binding of EGFα to EGFR on the cells is determined by the detection of $EGFR^+/Myc^+$ double-positive cells by flow cytometry.

For cell surface staining, cells were washed with PBS and blocked with Fc blocker (BD Biosciences, San Jose, CA). Fluorochrome labeled antibodies (EGFR, Annexin-V, CD45, CD3, CD4, CD8, CD11b, CD56, NKG2D, Myc) were obtained from BD Biosciences (Franklin Lakes, USA), added and stained

for 30 min, washed 3X with 2%FBS containing PBS and analyzed. All samples were analyzed on a BD FACS Aria flow cytometer.

In vitro co-culture killing assay. Ovarian cancer cells (SKOV3) were co-cultured with TALL-104 cells, at a different effector to target ratios (1:1, 2:1, and 5:1) for 2-3 days. Tumor cell lysis was monitored in real-time using real-time fluorescent microscopy (IncuCyte; Essen Biosciences). The cytotoxicity is reported by the percentage of viable cells/percentage of confluence remaining at the end of 48-hour co-culture.

Oncolytic virus and amplicon titration. Vero cells in 12-well plates were infected with serially diluted stocks in triplicates. The titer of Synco-2D is determined by plaque-forming units counted 24 to 48 h later. The amplicon titer is determined by counting the number of GFP^+ cells. In most stock preparations, the Synco-2D to amplicon ratio is approximately 8-10:1.

Western blot. Whole-cell lysates and supernatants from either transfected or infected cells were prepared and loaded onto an SDS-PAGE gel. After electrophoresis, the proteins were transferred to a membrane, which was first blocked with 5% skim milk for 1hr and then incubated with the diluted primary antibody for Myc-tag (1:2000) (Cell Signaling Technology, Danvers, MA) overnight. The membrane was washed with TBS-T three times and incubated with an HRP-labeled secondary antibody (anti-rabbit IgG, HRP linked Antibody) at 1:1000 dilution for 1hr at RT. The membrane was developed using the GE ECIL developer system.

Animal studies. Immune-competent female BALB/c mice (4 - 6 weeks old) were purchased from Charles River Laboratories. All animal experiments were approved by the University's Institutional Animal Care and Use Committee (IACUC). Right flanks of mice were shaved the day before tumor cell injection. The next day, $3x10^5$ CT26-EGFR cells were injected subcutaneously to the shaved right flank. Once the tumor volumes reached the approximate size of 6 mm in diameter, mice were randomized into different groups to receive either PBS control or Synco-2D treatment with or without the chimeric molecule-containing amplicons, at the dose of $5×10^6$ pfu Synco-2D per mouse. Three mice from groups receiving the treatment of PBS, Synco-2D GFP and Synco-2D TriCEP were euthanized on day 3 after virotherapy to collect tumor tissues for scRNA-seq or histology exam and spleens for other immune assays. The rest of the mice were kept for 2 to 3 weeks to monitor tumor growth by measuring two perpendicular tumor diameters with a caliper. Tumor volume was calculated by the formula: tumor volume (mm^3) = [length (mm)] × $[\text{width (mm)}]^2 \times 0.52$.

H & E staining and Immunohistochemistry. Tumor tissues were fixed and embedded in paraffin and sections were prepared. After de-paraffin and antigen retrieval, for H& E staining, the tissue sections were stained with Hematoxylin and Eosin following standard procedure. For IHC, the tissue sections were incubated in primary antibody GFP (Santa Cruz Biotech, Dallas, TX) overnight. After washing three times with PBS-T, the sections were incubated in secondary antibody for one h, and nuclei were stained with DAPI for 60 seconds. The slides were washed and analyzed under confocal or regular fluorescence microscopy.

Tumor dissociation and Single-cell processing. For scRNA-seq studies, the freshly collected tumors were immediately immersed in a tissue storage medium (Miltenyi, San Diego, CA) and kept at 4°C until ready for dissociation. Within 24 hours, tissues were processed to single-cell suspensions using the human tumor dissociation kit from Miltenyi and the gentleMACS apparatus and this was done by following the manufacturer's protocol. Single-cell suspensions were then stained with a fluorescently conjugated antibody specific to CD45 (BioLegend) for 30 min at 4ºC. The cells were washed with cell staining buffer (BioLegend) and $CD45^+$ live cells were sorted on a FACS Melody cell sorter (BD) into 2%FBS in PBS, which were kept on ice until the cells were further processed for scRNA-seq.

scRNA-seq Library preparation and sequencing. Cell suspensions were washed 2–4 times and manually counted twice to assure cell viability was >90% before loading onto the Chromium platform. The libraries were created from the cells by successfully capturing cells inside gel beads in emulsion (GEM) by passing cells through a microfluidic channel. Library fragmentation size and quantification were measured before sequencing to ensure that the cDNA has been fragmented and barcoded correctly. The cDNA libraries were assessed while using an Agilent Tapestation 4200 High sensitivity DNA tape. On the day of single-cell capture and library preparation, the cells were resuspended in PBS containing 0.04% bovine serum albumin (BSA) (Ambion, Foster City, CA) to a final concentration of 200 cells per µL. This cell suspension was used as an input for automated single-cell capture and barcoding using the 10X Genomics Full Chromium platform. Approximately 700 single cells were captured for each sample while using the 10X Genomics Single Cell 3' Chip at the university's Seq-N-Edit Core per standard protocols. Single-cell GEMs

were generated, and the single cells were uniquely barcoded. The cDNA was recovered and selected using DynaBead MyOne Silane Beads (Thermo Fisher Scientific, Carlsbad, CA) and SPRIselect beads (Beckman Coulter, Brea, CA). The sequencing libraries were generated and the quality was assessed using a high-sensitivity DNA tape on Tapestation 4200 (Agilent, Santa Clara, CA), and the fragments were counted with Qubit Fluorometer (Thermo Fisher Scientific, Carlsbad, CA) and Kapa Library Quantification Kit (Kapa Biosystems, Wilmington, MA) using the AriaMX instrument (Agilent, Santa Clara, CA). The libraries were sequenced using NextSeq 500 (Illumina, San Diego, CA) in stand-alone mode to obtain pair-end sequencing 26bp (read1) X 98bp (read2) and a single index 8bp in length.

Transcriptome Analysis. Single-cell sequencing data downstream analysis was performed on the Maxwell Cluster high-performance research computing center at the University of Houston, using the analytical program, Cell Ranger 4.0.0 Single Cell Analysis Pipelines (10X Genomics, Pleasanton, CA, USA). Raw base call files that were generated by NextSeq 500 were demultiplexed using the "cellranger mkfastq" function to generate FASTQ files. The reads were aligned to the mouse (mm10) genome using "cellranger count" function by STAR aligner[4]. The feature-barcode matrices across different samples were aggregated by "cellranger aggr" function, leading to an aggregated read count table.

Single-cell data analysis. After constructing the single-cell gene expression count matrix, we used the R package Seurat (v3.1.1)[5] for downstream analysis on R platform (v3.5.2). Transcription noise cells were firstly filtered by several criteria, including minimal expression of 200 genes per

cell and mitochondrial read percentage >10%. All cells passing quality control were merged into one count matrix and normalized and scaled using Seurat's NormalizeData and ScaleData functions. The reduced set of consensus highly variable genes was used as the feature set for independent component analysis using Seurat's RunPCA function. Cell clusters were identified using the shared nearest neighbor algorithm with a resolution parameter of 0.8. UMAP clusters of cells were identified based on the first 30 principal components.

To aid the assignment of cell type to clusters derived from unsupervised clustering, we performed cell-type enrichment analysis. Cell-type gene signatures obtained from BlueprintENCODE, Monaco Immune references from SingleR[6] and human cell landscape[7]. Mouse gene symbols were capitalized to map to human gene symbols. Each gene signature obtained from our clustering was statistically evaluated for overlap with gene signatures contained in these two resources.

Statistical analysis. All quantitative results are displayed as the mean ± S.D. The statistical difference between the two groups was compared using a Mann-Whitney U test or a Student's t-test. If more than two groups were compared, ANOVA was used. Statistical analysis was determined using Prism5 software (GraphPad Software, Inc., La Jolla, CA). A *p*-value of less than 0.05 was considered statistically significant.

References

1. Fu X, Tao L, Wu W, et al. Arming HSV-Based Oncolytic Viruses with the Ability to Redirect the Host's Innate Antiviral Immunity to Attack Tumor Cells. *Mol Ther Oncolytics* 2020;19:33-46. doi: 10.1016/j.omto.2020.09.002 [published Online First: 2020/10/08]
2. Nakamori M, Fu X, Pettaway CA, et al. Potent antitumor activity after systemic delivery of a doubly fusogenic oncolytic herpes simplex virus against metastatic prostate cancer. *Prostate* 2004;60(1):53-60.
3. Zhang X, De Alwis M, Hart SL, et al. High-titer recombinant adeno-associated virus production from replicating amplicons and herpes vectors deleted for glycoprotein H. *Hum Gene Ther* 1999;10(15):2527-37.
4. Du Y, Huang Q, Arisdakessian C, et al. Evaluation of STAR and Kallisto on Single Cell RNA-Seq Data Alignment. *G3: Genes|Genomes|Genetics* 2020;10(5):1775-83. doi: 10.1534/g3.120.401160
5. Butler A, Hoffman P, Smibert P, et al. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol* 2018;36(5):411-20. doi: 10.1038/nbt.4096 [published Online First: 2018/04/03]
6. Aran D, Looney AP, Liu L, et al. Reference-based analysis of lung single-cell sequencing reveals a transitional profibrotic macrophage. *Nat Immunol* 2019;20(2):163-72. doi: 10.1038/s41590-018-0276-y [published Online First: 2019/01/16]
7. Liao M, Liu Y, Yuan J, et al. Single-cell landscape of bronchoalveolar immune cells in patients with COVID-19. *Nat Med* 2020;26(6):842-44. doi: 10.1038/s41591-020-0901-9 [published Online First: 2020/05/14]