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Supplemental Information

Mesenchymal stromal cell delivery of oncolytic immunotherapy improves CAR-T cell antitumor activity

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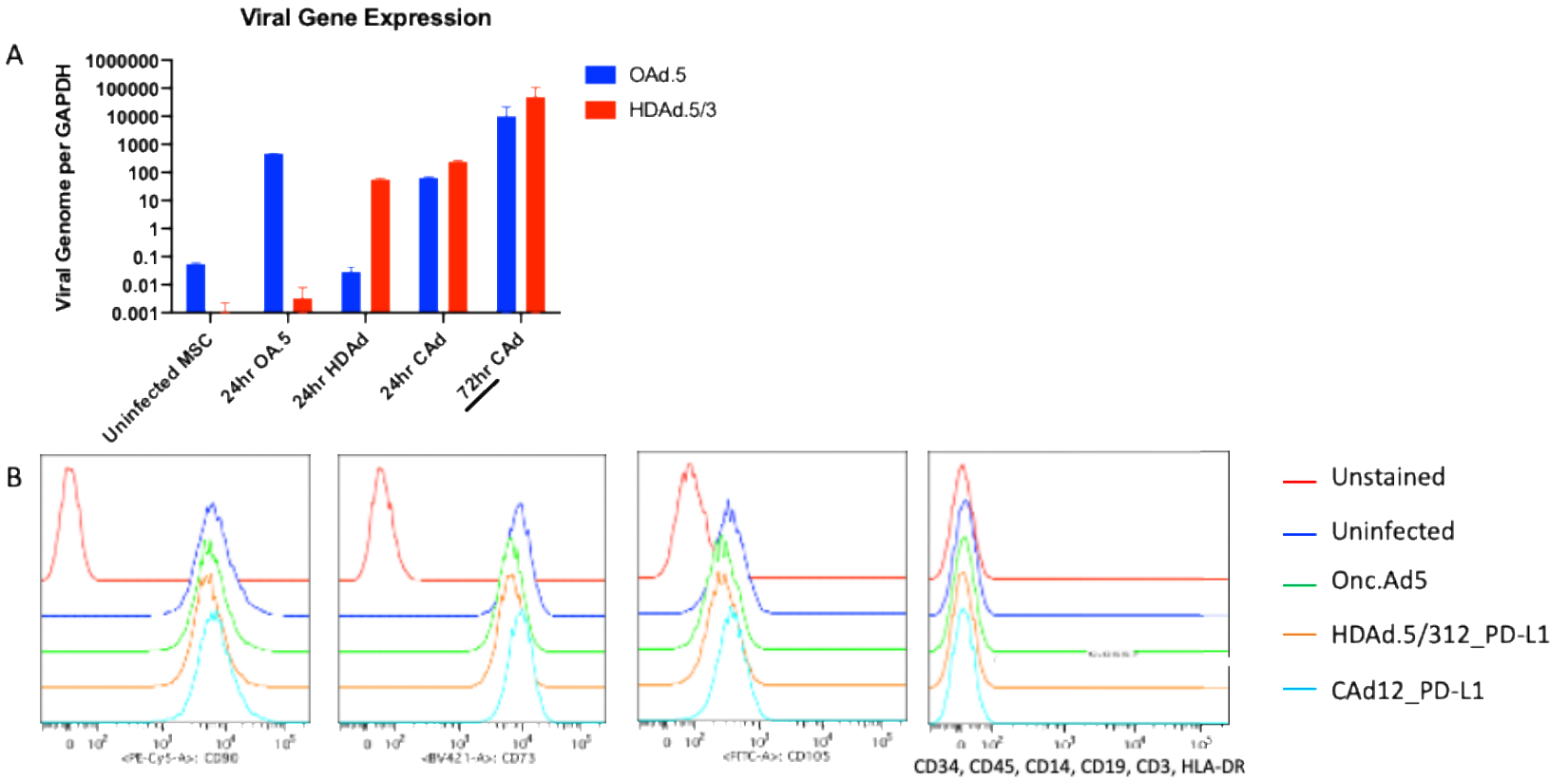
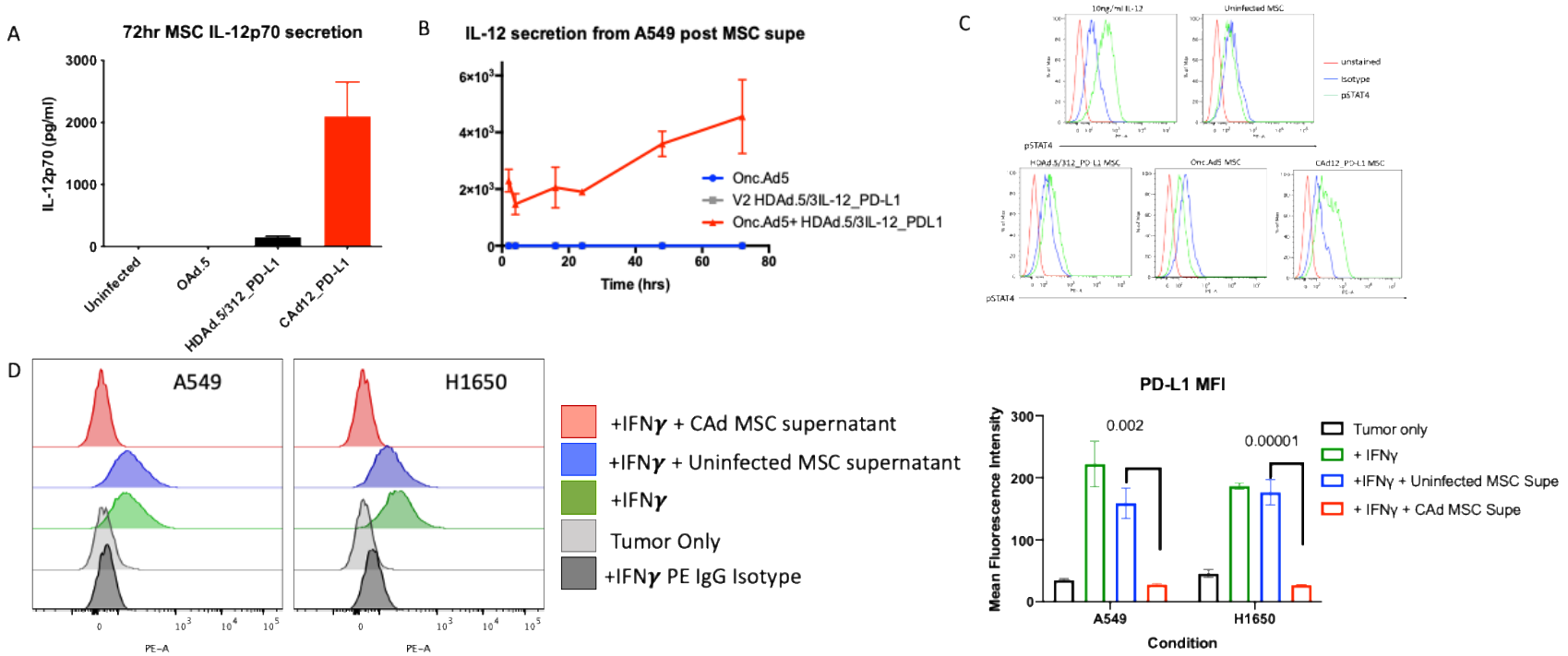
Supplemental Figure 1



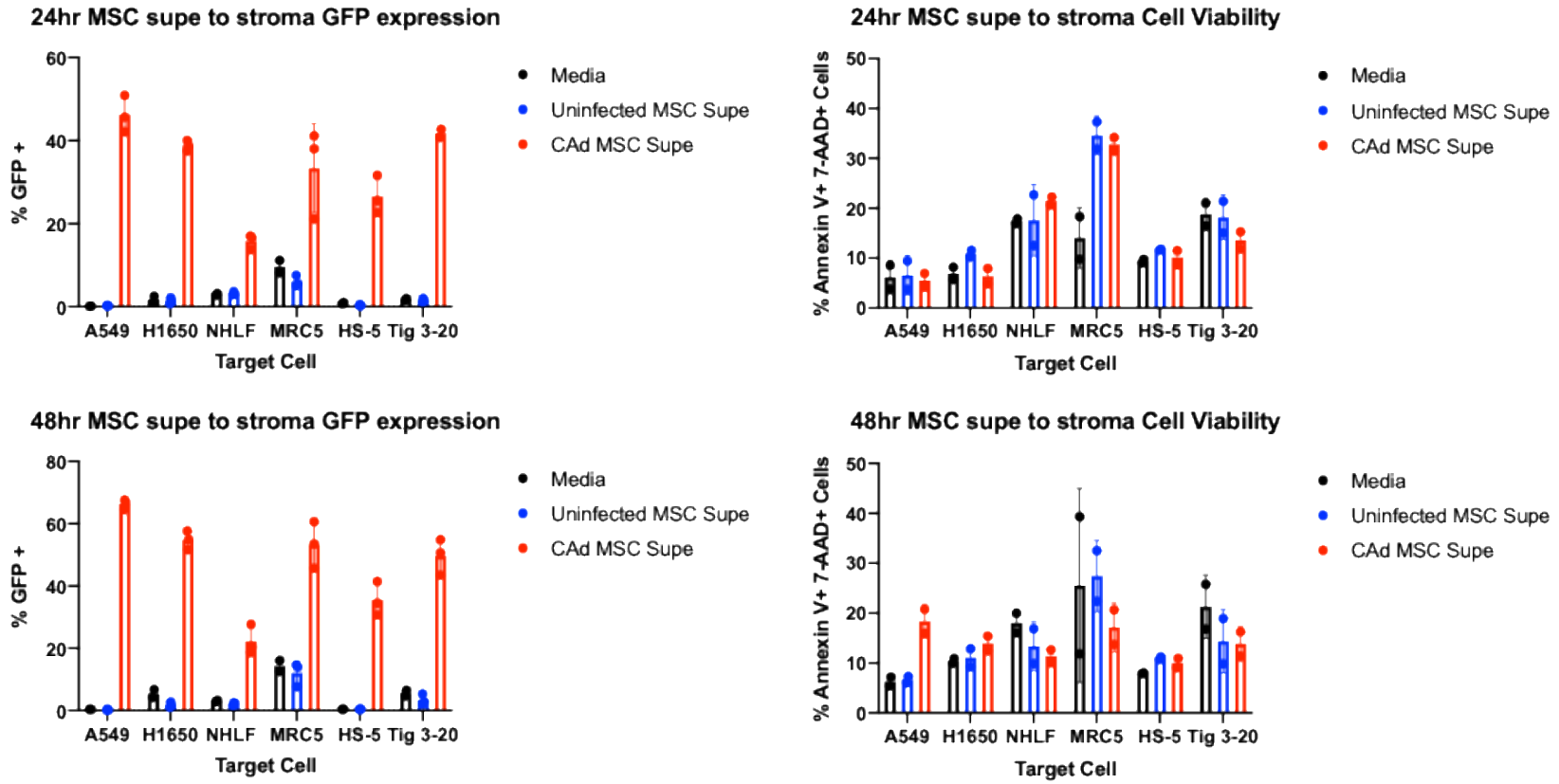
Supplemental Figure 1: **MSC reproduce virus and phenotype is not altered after Adenoviral infection.** A) MSCs were infected with 100vp Onc.Ad5+ 1000vp V2 HDAd.5/3 expressing IL-12 and anti-PD-L1. Viral genome expression of each virus was measured by qPCR and normalized to GAPDH at both 24hr and 72hrs post infection. B) MSC phenotype was analyzed 24hr post viral infection with Onc.Ad5 and HDAd.5/312_PD-L1 by flow cytometry. MSCs are defined by CD90, CD73, and CD105 positivity. MSCs also remained negative for CD45, CD14, CD34, and HLA-DR before and after infection.

Supplemental Figure 2



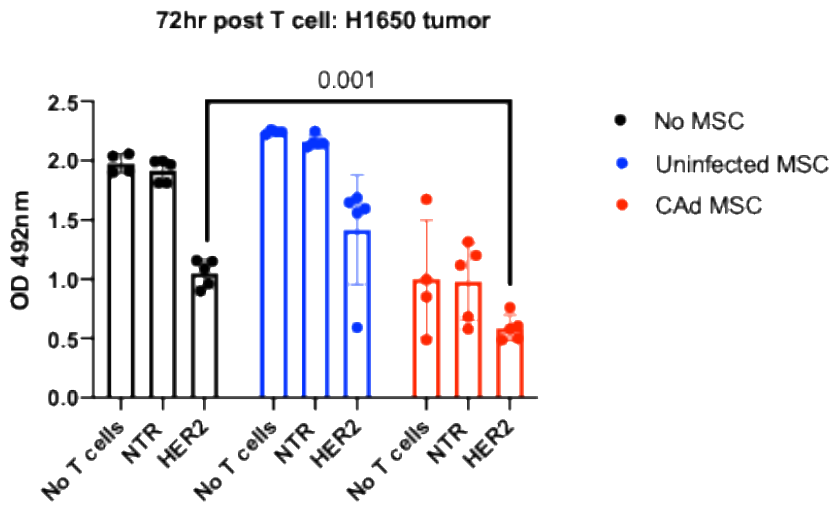
Supplemental Figure 2: **MSCs produce functional transgene products provided by helper dependent adenovirus** A) Supernatant was collected 72hrs post MSC infection and analyzed for IL-12p70 by ELISA B) 72hr MSC supe was applied to A549 cells and subsequent supes were collected at time points indicated. IL-12 secretion from A549 cells was measured by ELISA. C) 48hr supernatant from MSCs co-cultured with A549 tumor cells at a 1:5 ratio were applied to activated T cells for 30 minutes and then stained for phosphorylated STAT4 and analyzed by flow cytometry to determine IL-12 functionality. D) 48hr supernatant from three MSC donors (EY1, EY27, EY33) infected with CAd12_PD-L1 were applied to lung tumor cell lines stimulated with 10ng/ml IFNγ. Representative histogram for one MSC donor is shown. PD-L1 expression was measured by flow cytometry. The average Mean Fluorescence Intensity (MFI) between all three MSC donors are shown. Significance was determined by student t-test between uninfected MSC supernatant and CAd MSC produced supernatant.

Supplemental Figure 3



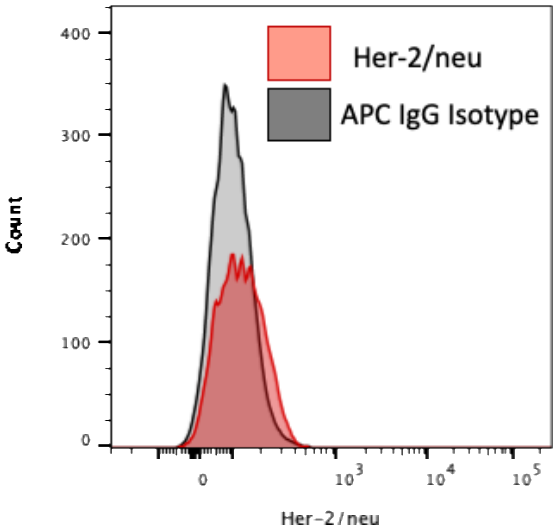
Supplemental Figure 3: **Stromal cells express HDAd transgenes but are resistant to oncolysis**. 24hr supernatant from three MSCs donors infected with 100vp/cell OAd.5 and 1000vp/cell HDAd.5/3 expressing GFP were applied to A549 and H1650 tumor cells, and Normal human lung fibroblasts (NHLF), MRC-5, HS-5 and Tig-3-20 fibroblasts. GFP expression was measure 24hr and 48hr post supernatant addition to indicate infection (left). Viability was measured over time by Annexin V+ conjugated to Pacfiic Blue and 7-AAD staining detected by flow cytometry (right).

Supplemental Figure 4



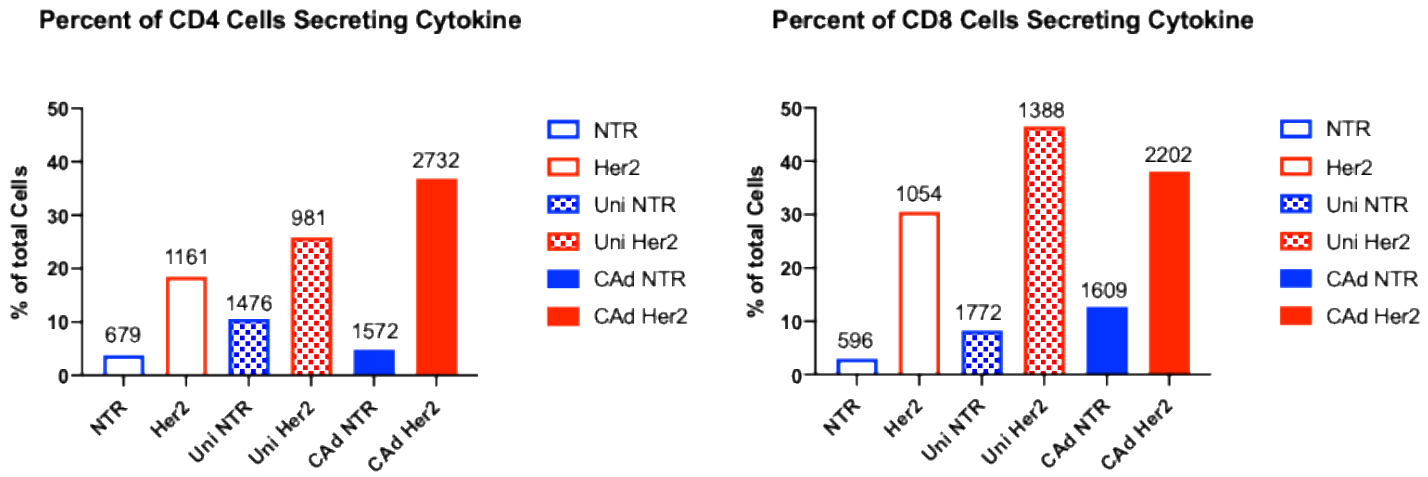
Supplemental Figure 4: **MTS assay to measure anti-tumor activity of CAd MSCs with HER.2 CAR-T cells.** $1x10^4$ H1650 tumor cells were added to a 96 well plate +/- $1x10^3$ Uninfected or CAd MSCs. MSCs and tumor were cultured at a 1:10 ratio for 48hrs followed by $2.5x10^3$ non-transduced or HER.2 specific CAR-T cells. Cell viability was analyzed by MTS assay according to the manufacturer's protocol (Promega, Cell Titer Glo). Significance was determined by student t-test.

Supplemental Figure 5



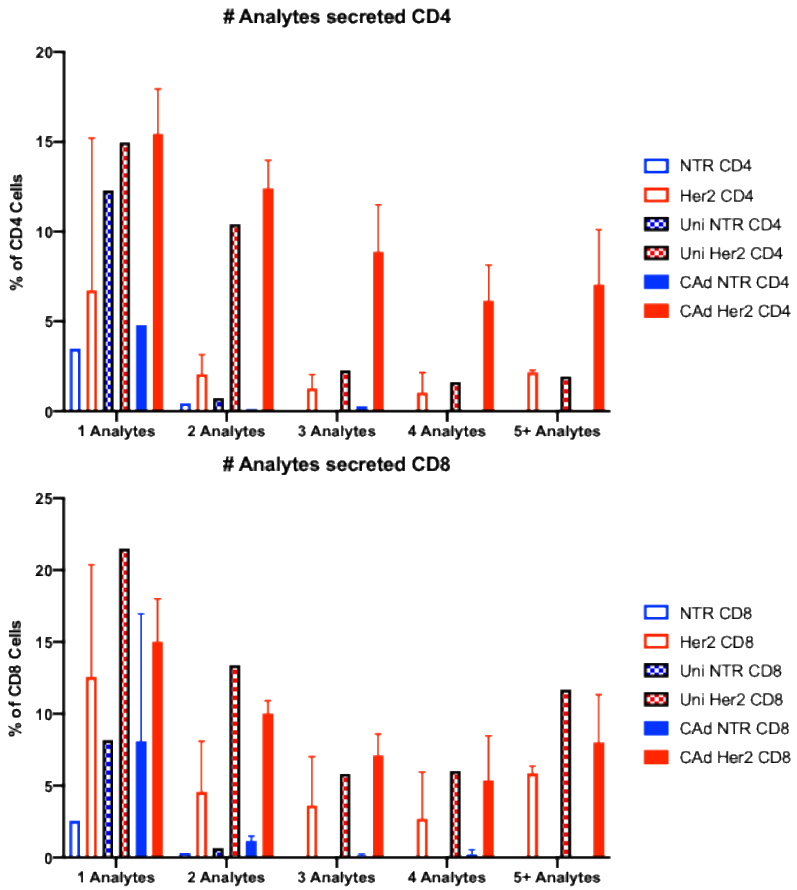
Supplemental Figure 5: **Her-2/neu expression on healthy MSC donor**. EY11 MSCs derived from healthy bone marrow and cultured for 4 passages were assessed for Her-2/neu surface expression by flow cytometry.

Supplemental Figure 6



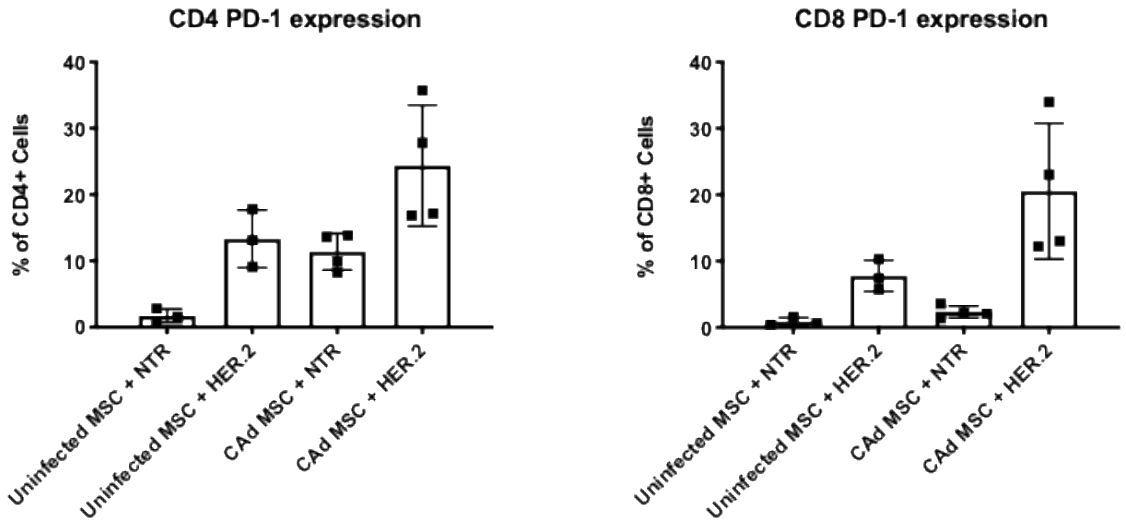
Supplemental Figure 6: **CAd MSCs enhance CD4 T cell cytokine secretion**. Non-transduced or HER.2 CAR-T cells were cultured with tumor only or with tumor cultured with uninfected or CAd MSCs for 20hrs. T cells were then isolated and separated into CD4 and CD8 populations. Single cell cytokine secretion was measured by Isoplexis. Number above each bar indicates the total number of cells analyzed in each condition between three donors. The bar graph shows the frequency of the total population secreting cytokine.

Supplemental Figure 7



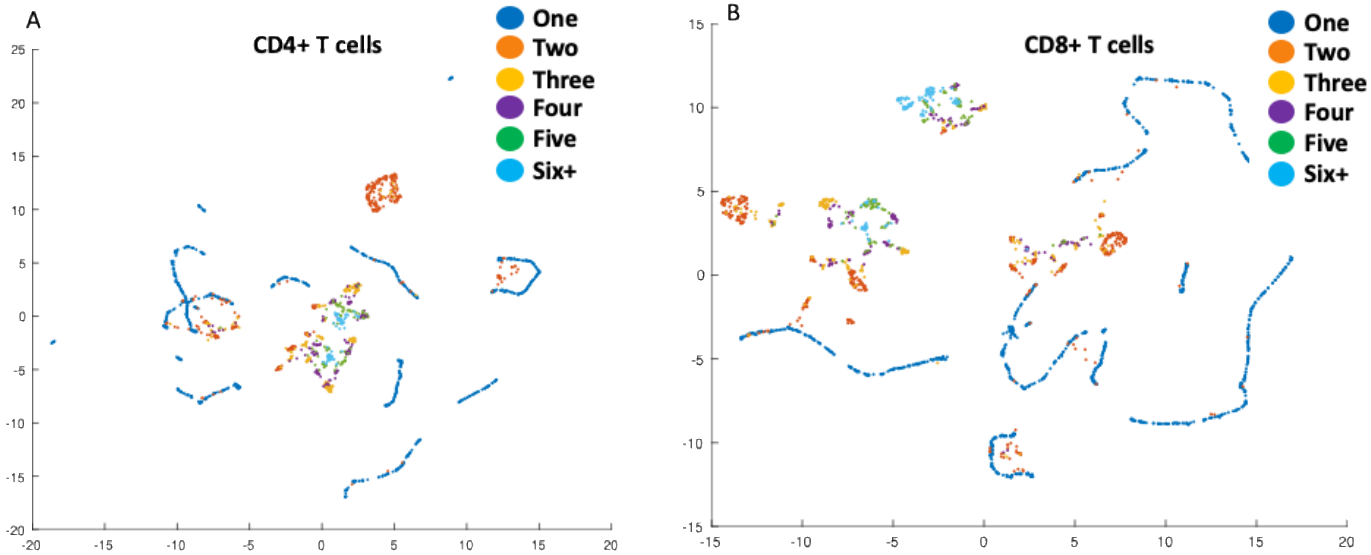
Supplemental Figure 7: **Polyfunctionality of T cells.** Number of analytes secreted according to treatment of 32-multiplexed Isoplexis single cell secretion system.

Supplemental Figure 8



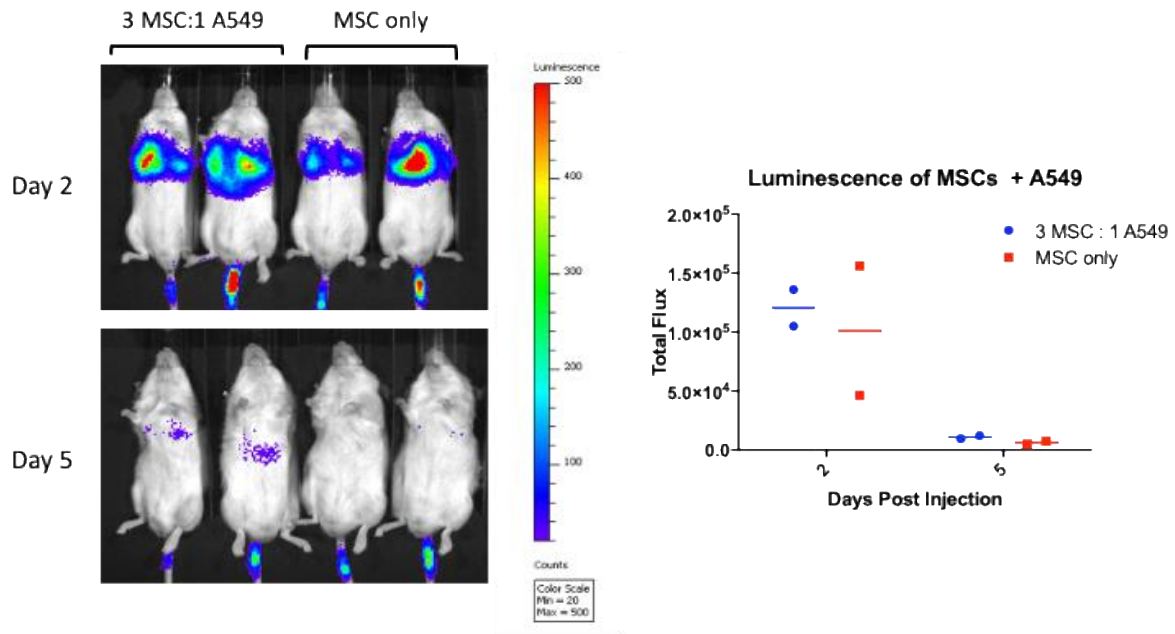
Supplemental Figure 8: **Increased PD-1 expression on CD4 (left) and CD8 (right) T cells cultured with CAd MSCs**. $2x10^5$ GFP+A549 tumor cells were culture with $2x10^4$ uninfected or CAd infected MSCs (10 tumor: 1MSC) for 48hrs. $5x10^4$ non-transduced of HER.2 specific CAR-T cells were then cultured with tumors and MSCs for 72hrs. T cells were isolated and analyzed for cell surface PD-1 expression by flow cytometry.

Supplemental Figure 9



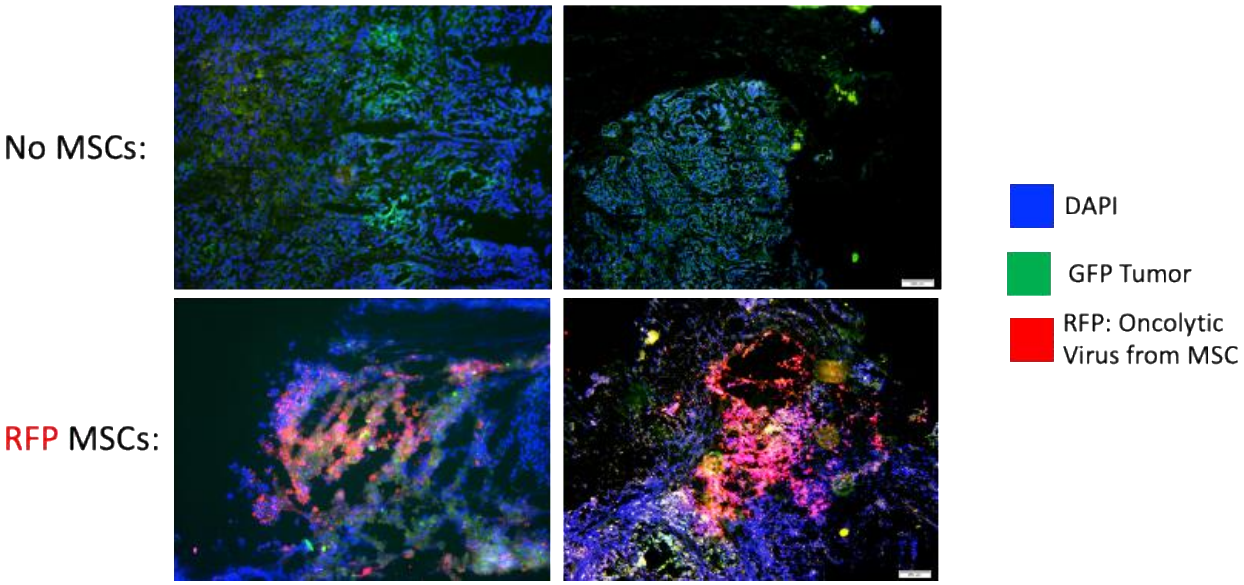
Supplemental Figure 9: **T cells cluster in similarity by number of cytokines secreted**. Single cell cytokine secretion was measured by the Isoplexis platform and Umap analysis was carried out on MatLab software. CD4 (A) and CD8 (B) T cells cluster based on polyfunctionality.

Supplemental Figure 10



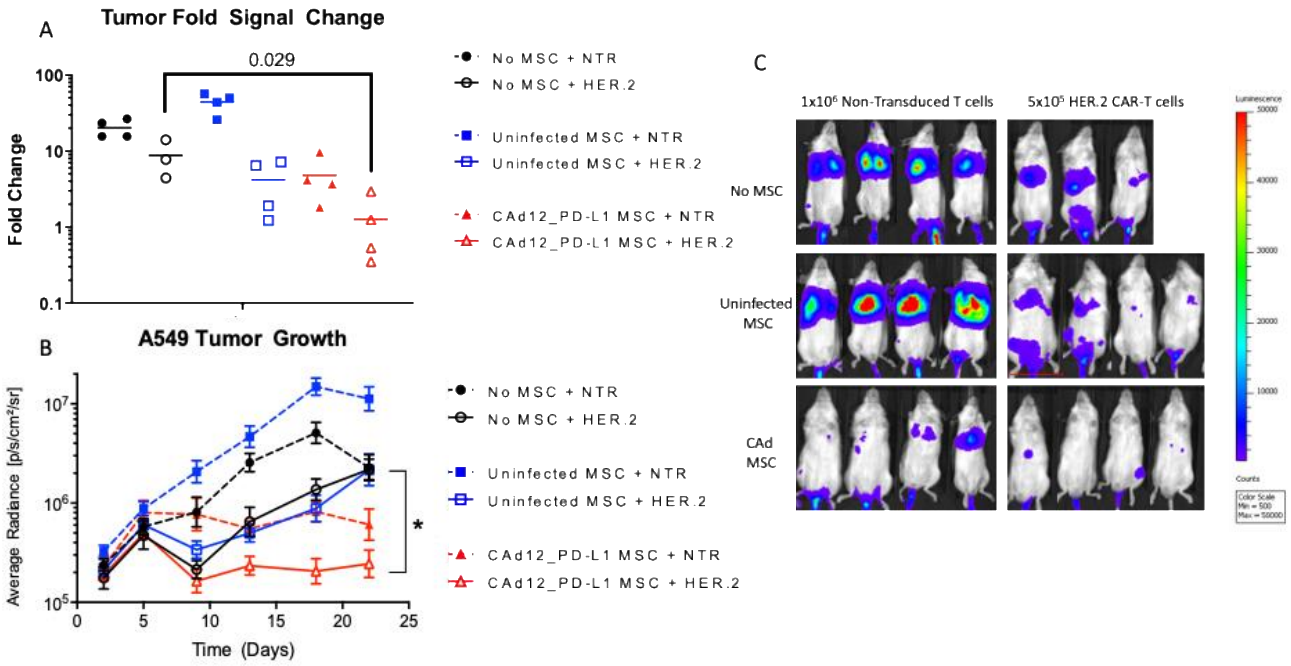
Supplemental Figure 10: **MSCs home to the lungs/site of tumor and only remain viable for 5 days post infection**. NSG mice were injected intravenously with $2x10^6$ uninfected MSCs expressing firefly luciferase with or without unlabeled A549 tumor cells at a ratio of 3 MSCs to 1 tumor. Images were acquired by IVIS imaging on Day 2 and Day 5 post injection. Right: Signal quantified from the lungs of mice post infusion.

Supplemental Figure 11



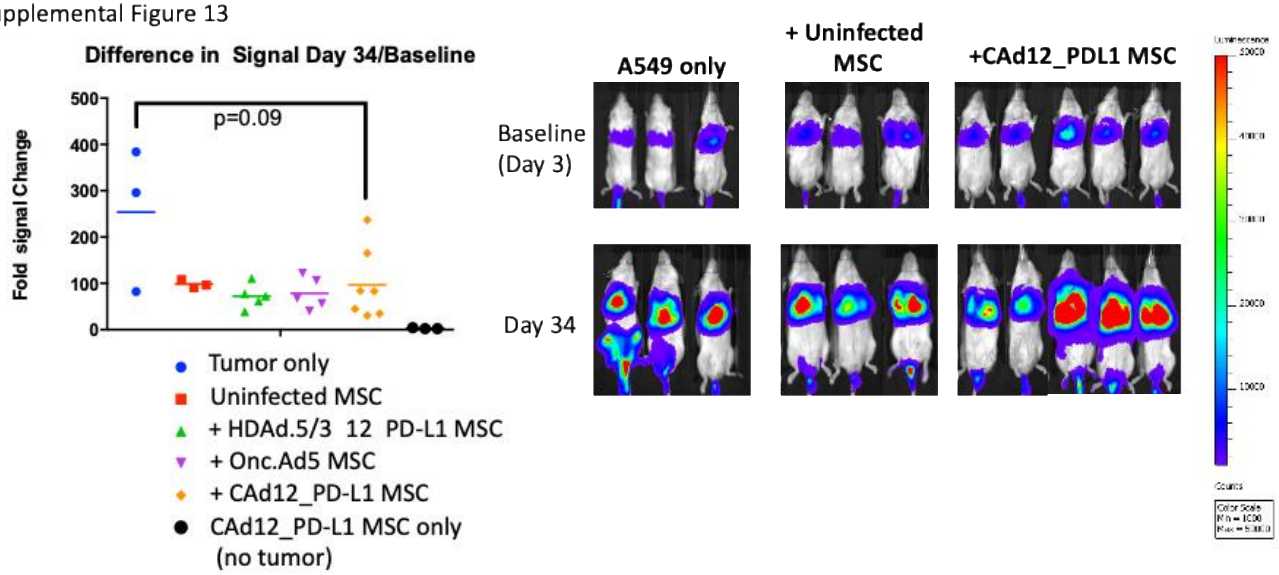
Supplemental Figure 11: **Virus detection in tumor and stromal tissue**. Lung tumor tissues were harvested on Day 5 post MSC infusion. Mice received either $2x10^6$ uninfected MSCs or OAd expressing RFP MSCs. A549 tumor cells expressed GFP-Ffluc and sections were stained for DAPI to indicated nuclear stain. Tissues with no MSCs show both tumor (GFP, DAPI+) and stromal cells (non GFP, DAPI+). Mice treated with CAd RFP MSC show tumor (GFP, DAPI+) and stromal cells (non-GFP, DAPI+) express RFP indicating both cell populations are infected with the CAd vector.

Supplemental Figure 12



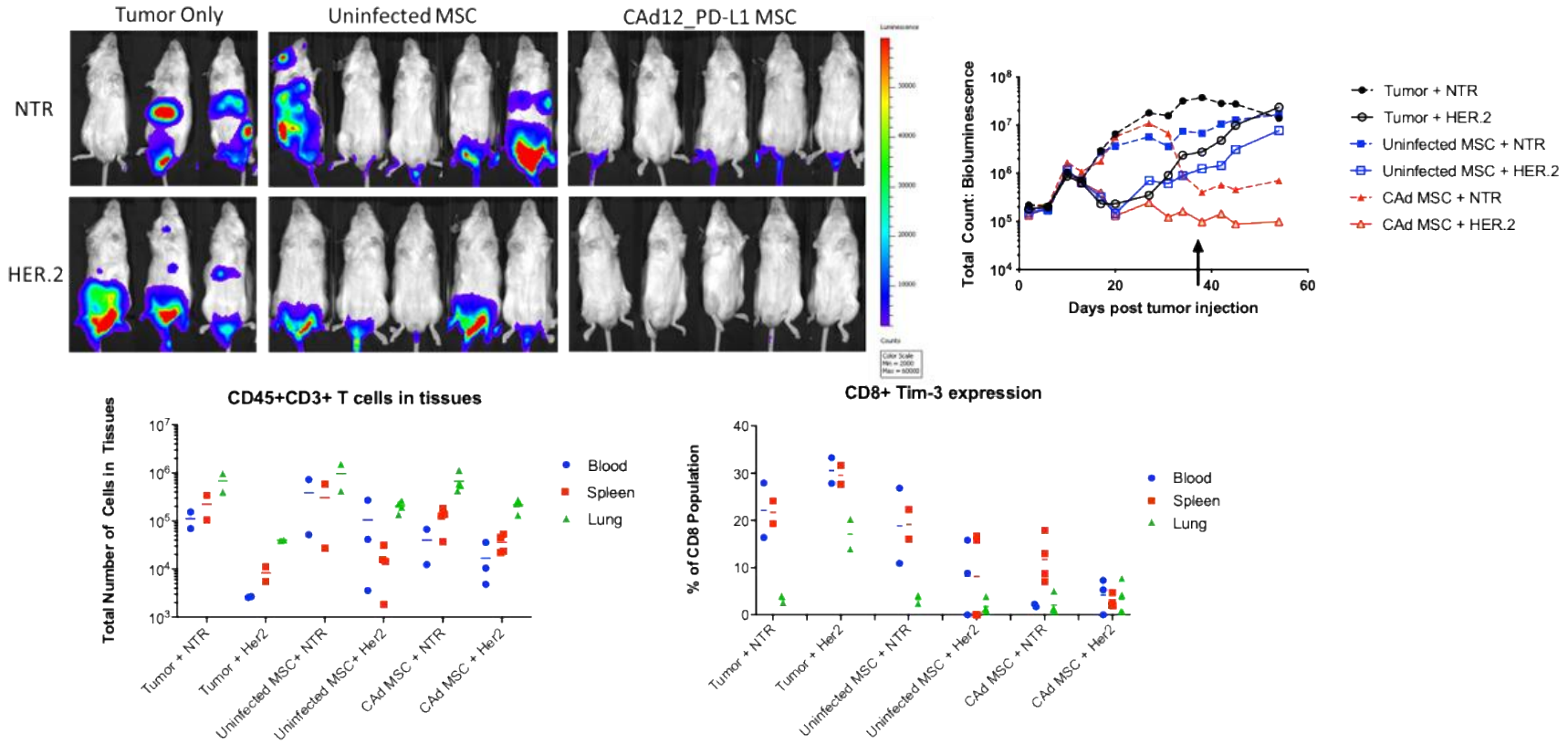
Supplemental Figure 12: **CAd12_PD-L1 MSC improves anti-tumor activity of HER.2 CAR-T cells in A549 tumor model:** NSG mice were engrafted with A549 GFP-Ffluc labeled tumor cells intravenously followed by IV infusion of $2x10^6$ uninfected or CAd12_PD-L1 MSC on Day 3. On Day 6, mice were infused with $5x10^5$ NTR or HER.2 specific CAR-T cells. $1x10^6$ NTR cells were used for A549 tumor model control. Fold change was determined from baseline imaging on Day 3 post tumor engraftment. P valued was determined by Mann-Whitney U test. B) Tumor growth was monitored by *in vivo* imaging to measure bioluminescence over time. * indicates p=0.01 as determined by student t-test, n=4-5 per group. C) Bioluminescent animal images are shown for last day of measurement.

Supplemental Figure 13



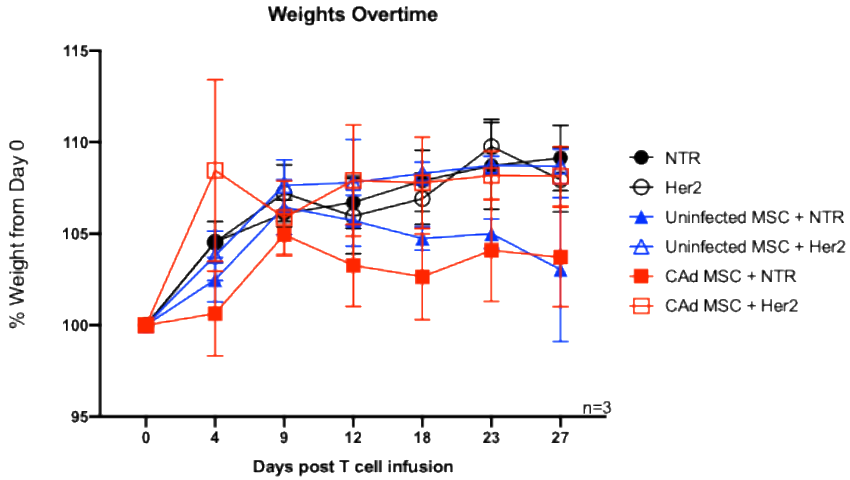
Supplementary Figure 13: **CAd MSCs only have minimal anti-tumor activity in an orthotopic lung tumor model**. 2x10^6 A549 GFP Ffluc expressing tumor cells were injected via tail vein. Day 4 post tumor establishment, 2x10^6 uninfected MSC or infected MSCs were administered intravenously by tail vein. Tumor bioluminescence was detected by IVIS imaging system. P value determined by student's t test.

Supplemental Figure 14



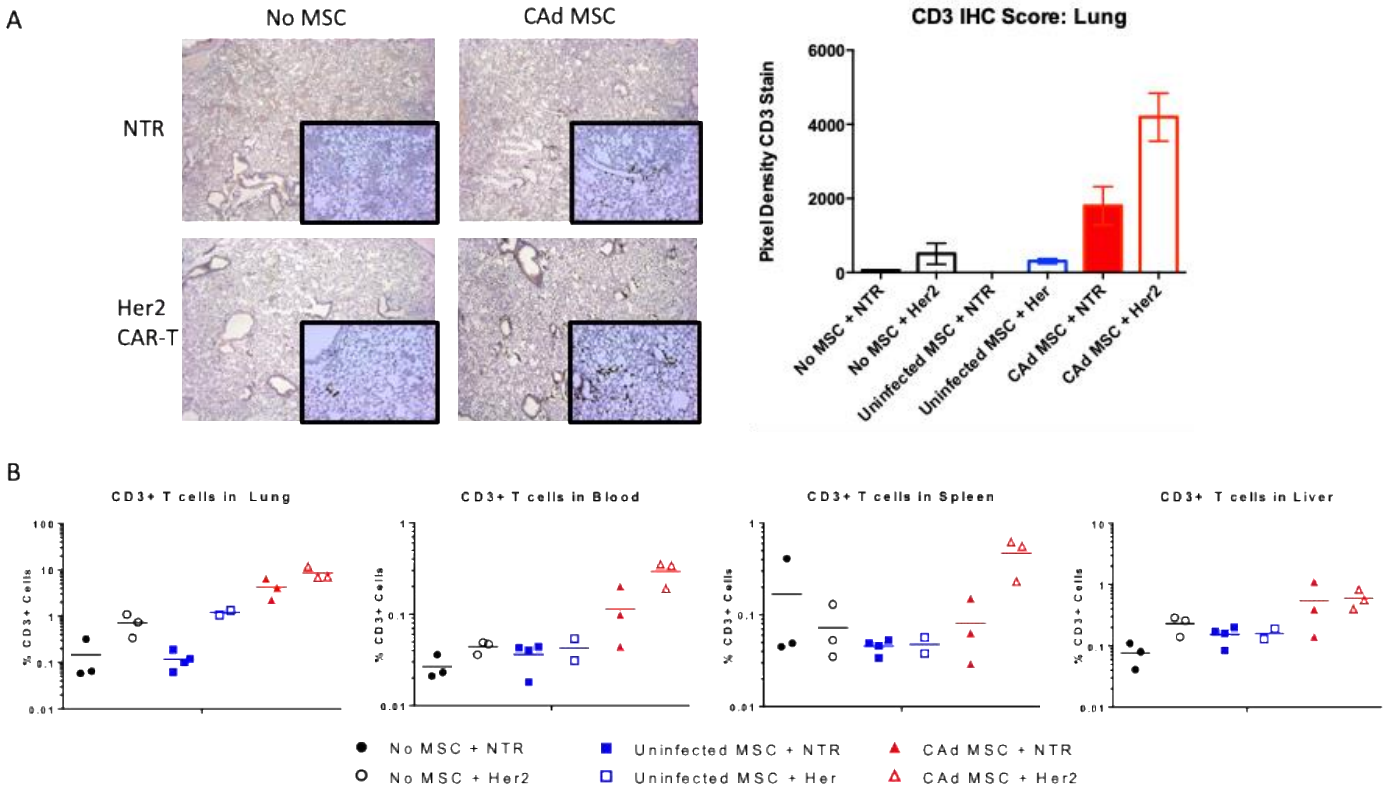
Supplemental Figure 14: **CAd12_PD-L1 MSCs with CAR-T cells overcome tumor re-challenge.** NSG mice were engrafted with GFP-Ffluc labeled A549 cells intravenously followed by IV infusion of $1x10^6$ uninfected or CAd12_PD-L1 MSC on Days 3, 7, &11. $3x10^6$ HER.2 or NTR T cells were infused on Day 14 and mice were re-challenged with $2x10^6$ tumor cells on Day 38 (arrow). Tumor growth was monitored by in vivo imaging to measure bioluminescence. Image is Day 54 post tumor engraftment and tissues were stained for T cells on Day 65, analyzed by flow cytometry.

Supplemental Figure 15



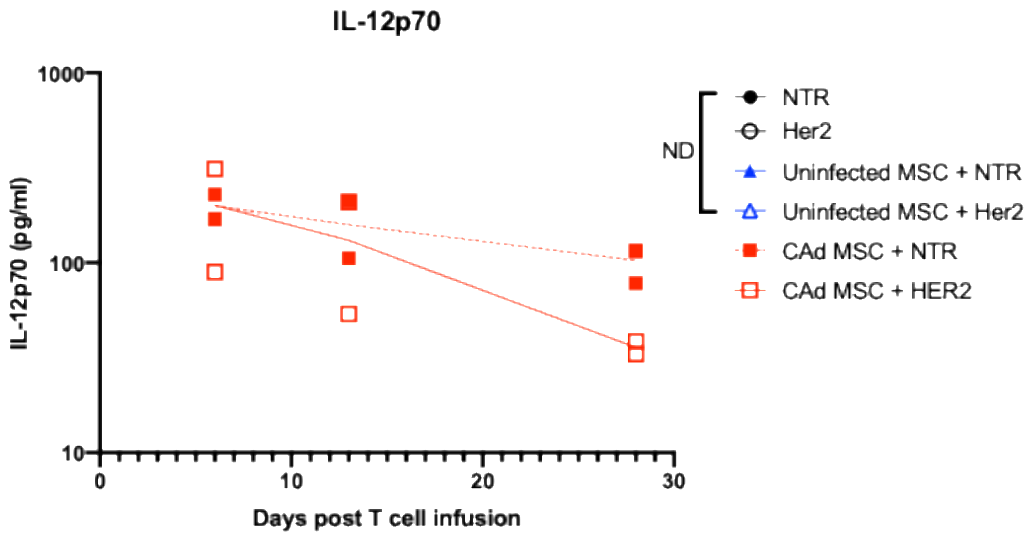
Supplemental Figure 15: **No toxicities observed with combination treatments in vivo**. Weights of the animals were monitored and normalized to 100% at Day 0 for tumor engraftment.

Supplemental Figure 16



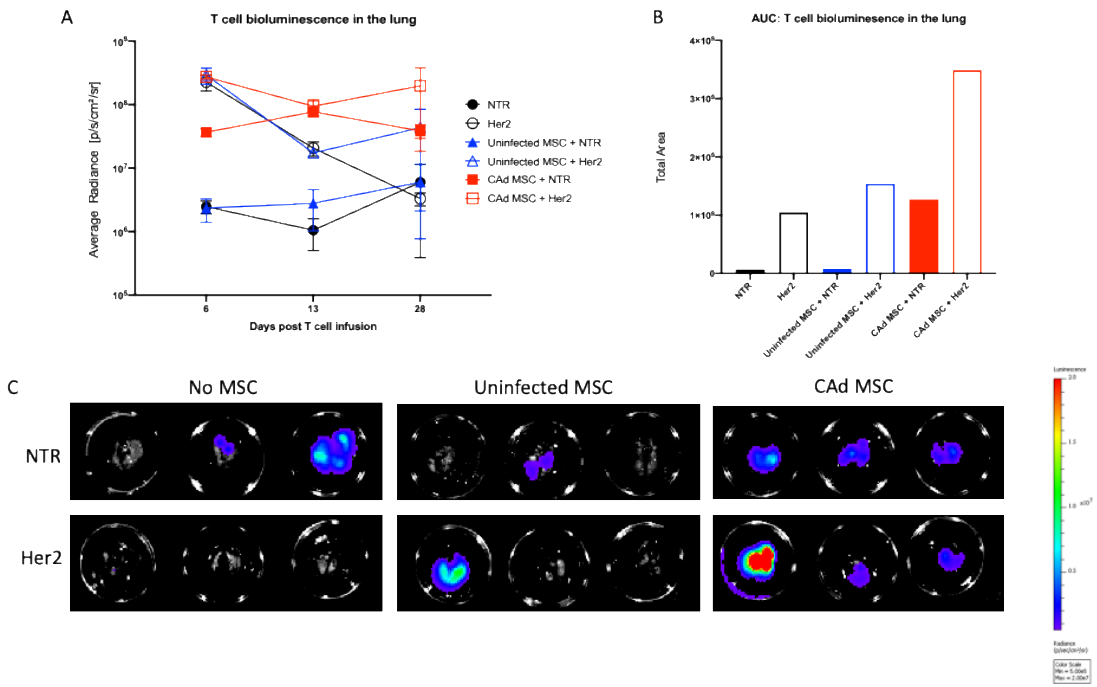
Supplemental Figure 16: **CAd12_PD-L1 MSC and HER.2 CAR-T cells increase the number of CD3+ T cells in the lungs and peripheral tissues.** A) Day 18 post tumor engraftment, lung tissues were isolated and processed for IHC staining of CD3. CD3 intensity was scored and quantitated n = 2 mice/group. B) Tissues were isolated and process on Day 18 post tumor engraftment to determine CD3+ T cell population in each tissue by flow cytometry.

Supplemental Figure 17



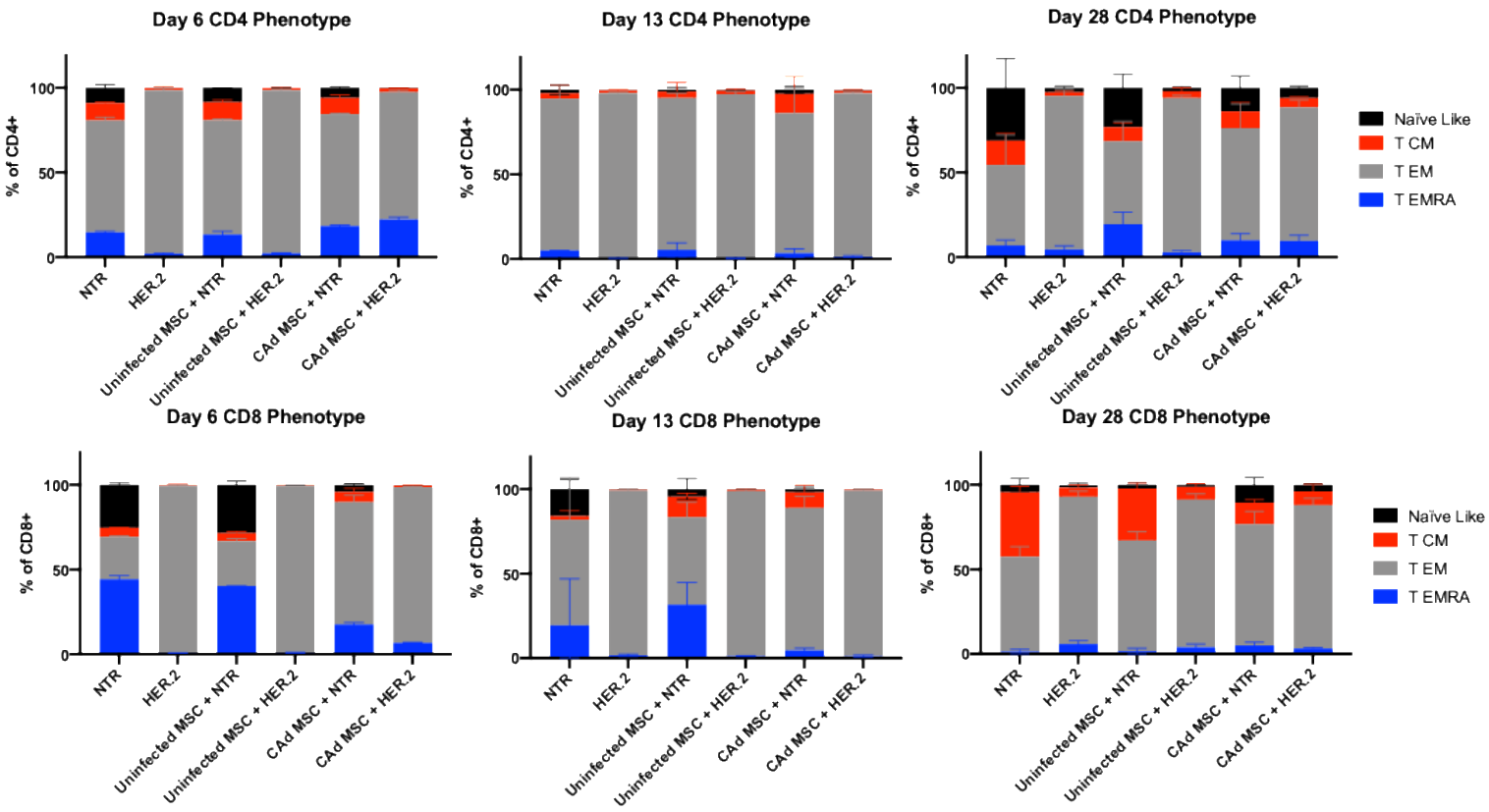
Supplemental Figure 17**: IL-12 detection in the plasma of mice on Day 6, 13, and 28 post T cell infusion**. Blood was collected from each mouse at the time of sacrifice. Plasma was isolated and analyzed for IL-12 detected by ELISA. Each point represents a single animal with detectable levels. ND = not detected.

Supplemental Figure 18



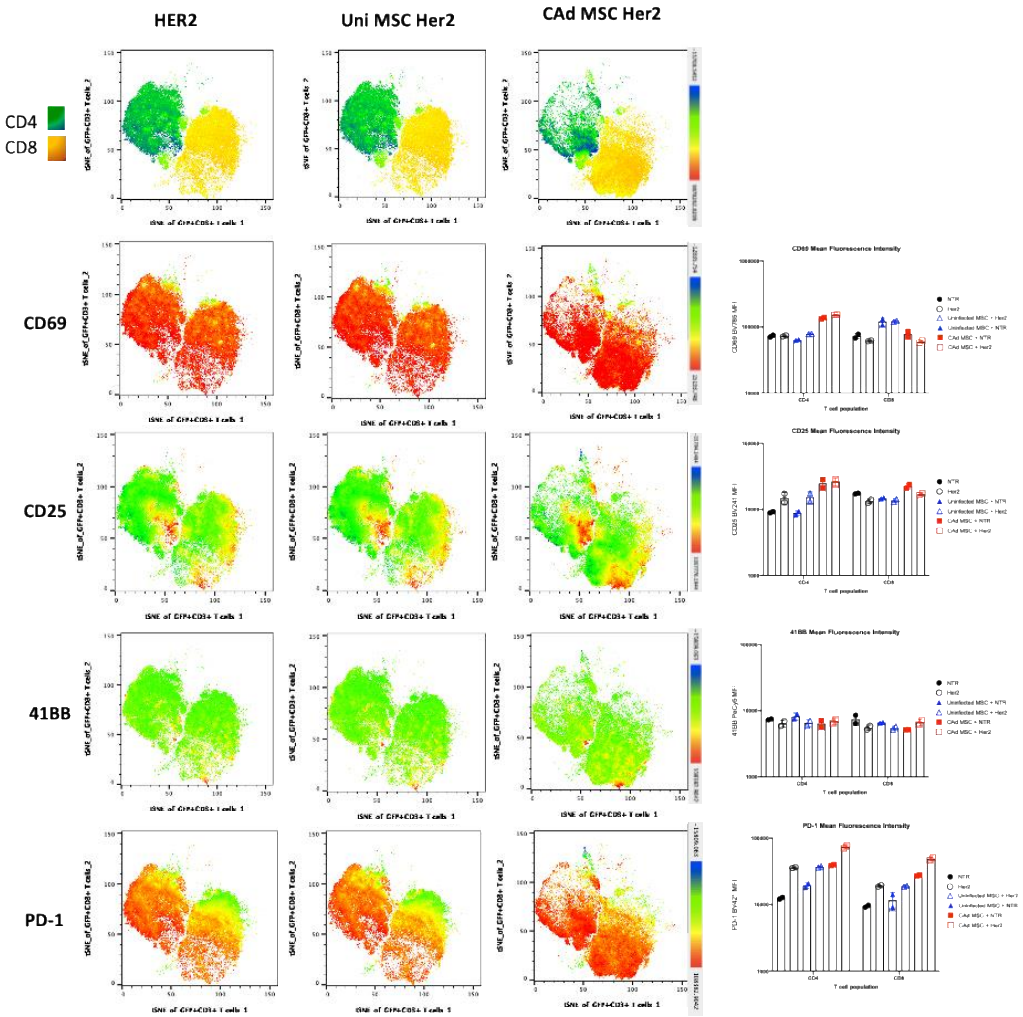
Supplemental Figure 18: **T cell signal detected in the primary tumor site over time.** A) Bioluminesence signal of T cell infiltrating into the lungs of mice overtime. n=2 mice per group on days 6 and 13 and n=3 mice per group on day 28. B) Area under the curve quantified from A. C) Bioluminescent images of lung tissues ex vivo on Day 28.

Supplemental Figure 19



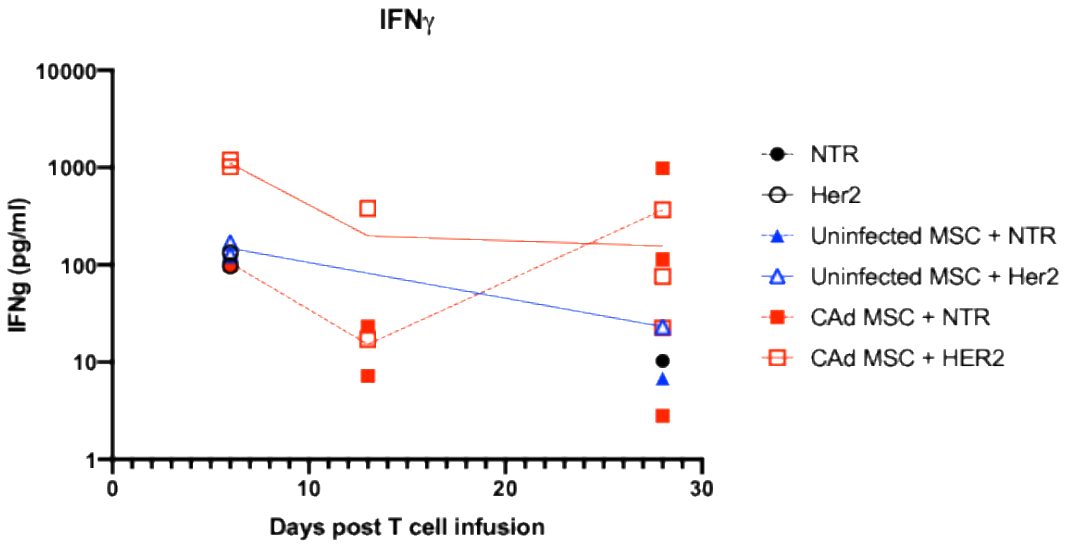
Supplemental Figure 19: **Combination treatment did not significantly alter T cell memory subsets**. Human T cells were isolated from the lung tissues at designated time points post T cell infusion and stained for memory T cell markers by CCR7 (CD197) and CD45RA. Two mice were averaged for Days 6 and 13 and three mice were averaged on Day 28.

Supplemental Figure 20



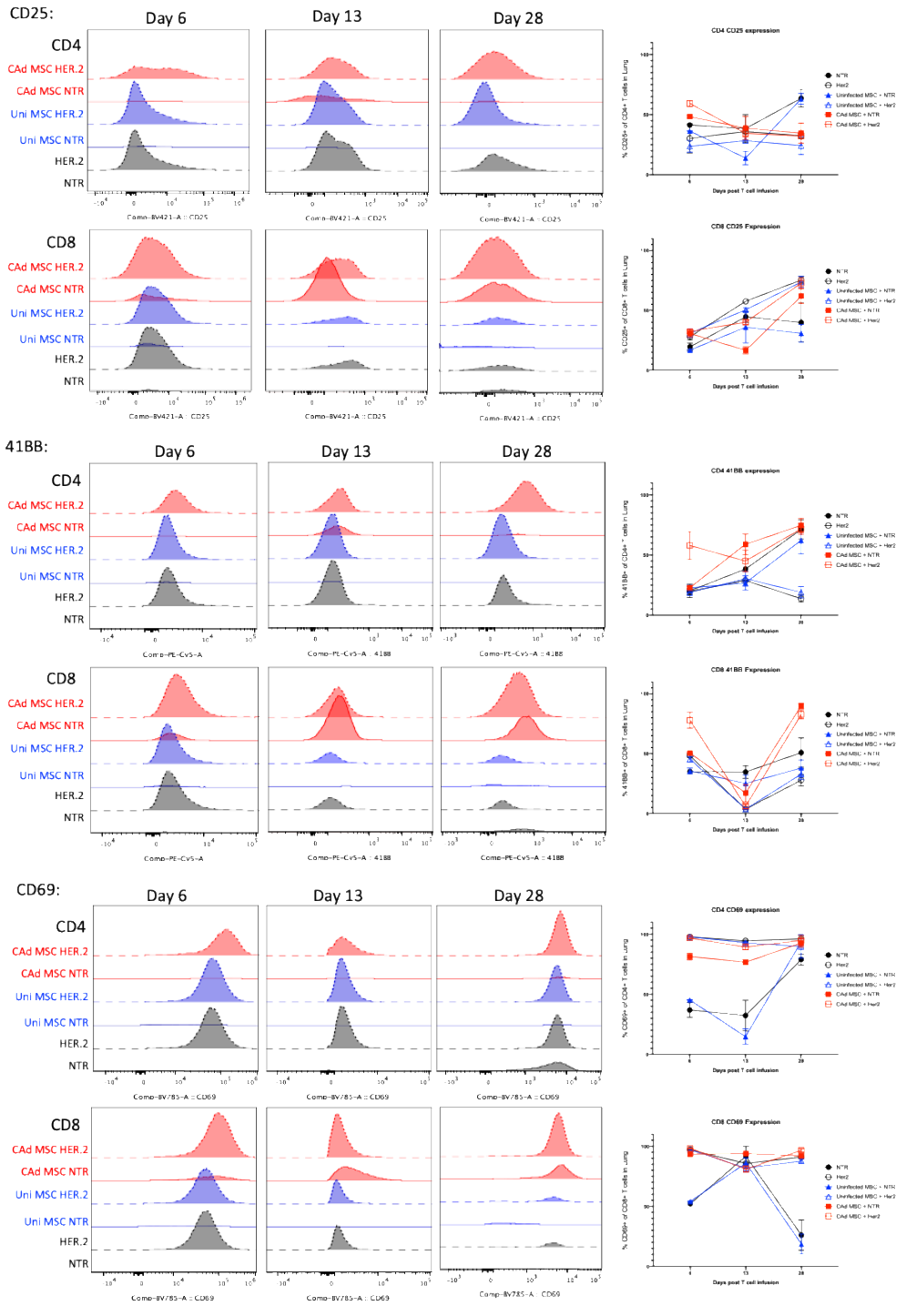
Supplemental Figure 20: **tSNE representation of activation markers on HER.2 CAR-T cells Day 6 post infusion**. Total CD3+ T cell populations are shown with CD4 and CD8 marker indication in the first row. Mean fluorescence intensity if shown to the right activation marker per animal (n=2).

Supplemental Figure 21



Supplemental Figure 21: **IFNγ detection in the plasma of mice on Day 6, 13, and 28 post T cell infusion**. Blood was collected from each mouse at the time of sacrifice. Plasma was isolated and analyzed for IFNγ detected by ELISA. Each point represents a single animal with detectable levels.

Supplemental Figure 22



Supplemental Figure 22: **CAd MSCs enhance T cell activation in vivo:** T cells were isolated from tissues on Day 6, 13, and 28 post infusion. CD25 and 41BB expression for one representative mouse is shown in the histogram measured by flow cytometric analysis. The average percent of CD25 and 41BB expression for CD4 and CD8 T cells is shown in the line graphs to the left.

Supplemental Video 1: MSCs were infected with 100vp/cell OAd expressing RFP and 1000vp/cell HDAd expressing GFP. Supernatant was collected 72hrs later and applied to A549 (left) and H1650 (right) cell lines. Images were acquired every 2hrs by Incucyte Live Cell image analysis for 3 days.

Supplemental Video 2: Confocal imaging to visualize MSC incorporation of tumor spheroid and oncolytic virus spread. 2000 GFP+ H1650 or A549 lung tumor cells were seeded to an agarose coated 96 well plate. 200 uninfected or CAd infected were stained with efluor 670 and added 48hrs after spheroid generation. CAd infected MSCs were infected with 100vp/cell OAd expressing RFP and 1000vp/cell HDAd expressing IL-12 and PD-L1 blocker. Images were collected at time of MSC addition and then every 24hrs for 4 days on a GE Healthcare DeltaVision LIVE High Resolution Deconvolution Microscope.

Supplemental Video 3: CAd MSC and HER.2 CAR-T cells target tumor spheroids. 2000 GFP+ H1650 lung tumor cells were seeded to an agarose coated 96 well plate. 200 MSC were added 48hrs later followed by 50 non-transduced or HER.2 specific CAR-T cells. GFP fluorescence indicated viable tumor with RFP Annexin V+ staining used to show tumor cell apoptosis. Images were acquired every 2hrs for 5 days on the Incucyte.