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Supplemental Information

Analysis and Augmentation of the Immunologic Bystander Effects of CAR T Cell Therapy in a Syngeneic Mouse Cancer Model

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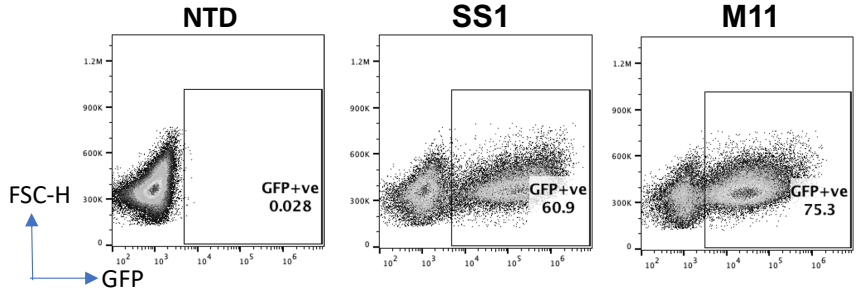
Supplemental Figures and Legends

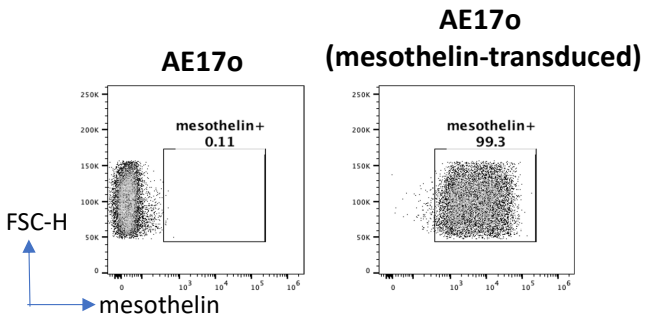
Figure S1

A.

B.



C.



D.

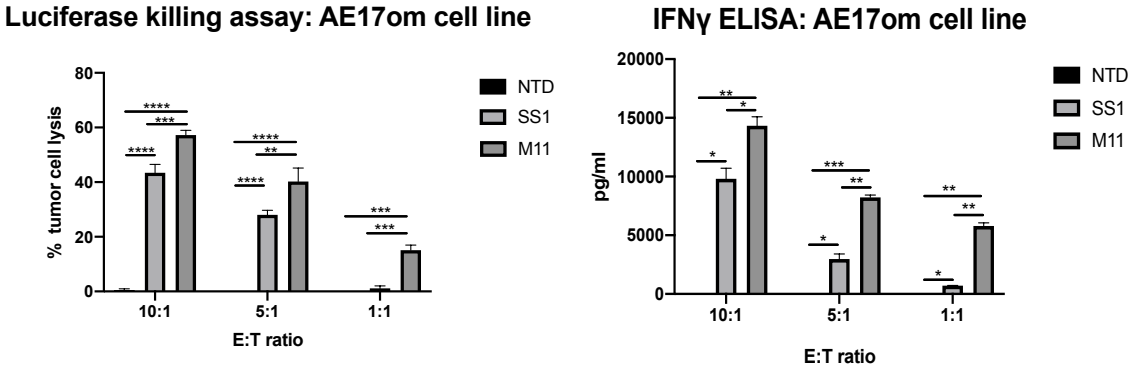


Figure S1. Development and characterization of murine CAR T cells. A. M11 CAR design. The VH and VL sequences of the M11 mesothelin-specific scFv were fused with mouse CD8a hinge, CD8a transmembrane domain, and two mouse ICDs derived from 4-1BB and CD3z. This CAR was subcloned into the MIGR1 retroviral vector, which also expresses GFP, using an internal ribosomal entry site. **B. Transduction efficiency of murine T cells.** Flow tracings showing transduction efficiency of murine T cells engineered either with the control SS1 CAR or the developed M11 CAR. Non-transduced (NTD) murine T cells were used as negative control. SS1 CAR T cells were stained with a mesothelin Gab-Fab antibody directly conjugated with FITC (1:25 dilution); M11 CAR T cells were analysed based on their GFP expression. Analysis was performed via flow cytometry on d3 post T cell transduction. **C**. Flow tracings of murine MM cell line AE17o and AE17om, demonstrating stable expression of mesothelin only in the AE17om cells. **D. In vitro efficiency of M11 CAR T cells.** Cytotoxicity of M11 CAR T cells was assessed with a luciferase killing assay **(left graph).** 30,000 tumor cells were seeded in triplicate wells of a 96-well plate and, following adherence after about 4 hrs, NTD, SS1 CAR or M11 CAR T cells were added to wells at E:T ratios of 10:1, 5:1 and 1:1. After overnight incubation, supernatants were collected and cytotoxicity of CAR T cells was measured by killing assay, using the Promega luciferase assay kit. Data showed that NTD cells had no killing activity against tumor cells. M11 CAR T cells demonstrated significantly better cytotoxicity against tumor cells in all E:T ratios compared to the control SS1 CAR T cells. Samples n= 3 per group. Statistics by 2-way ANOVA (**$p<0.01$, ***$p<0.001$, ****$p<0.0001$). IFNγ production correlated with the cytotoxicity data, as measured in the co-cultures' supernatants by a mouse IFNγ ELISA kit **(right graph).** Samples n= 3 per group. Statistics by 2-way ANOVA (**$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

Figure S2

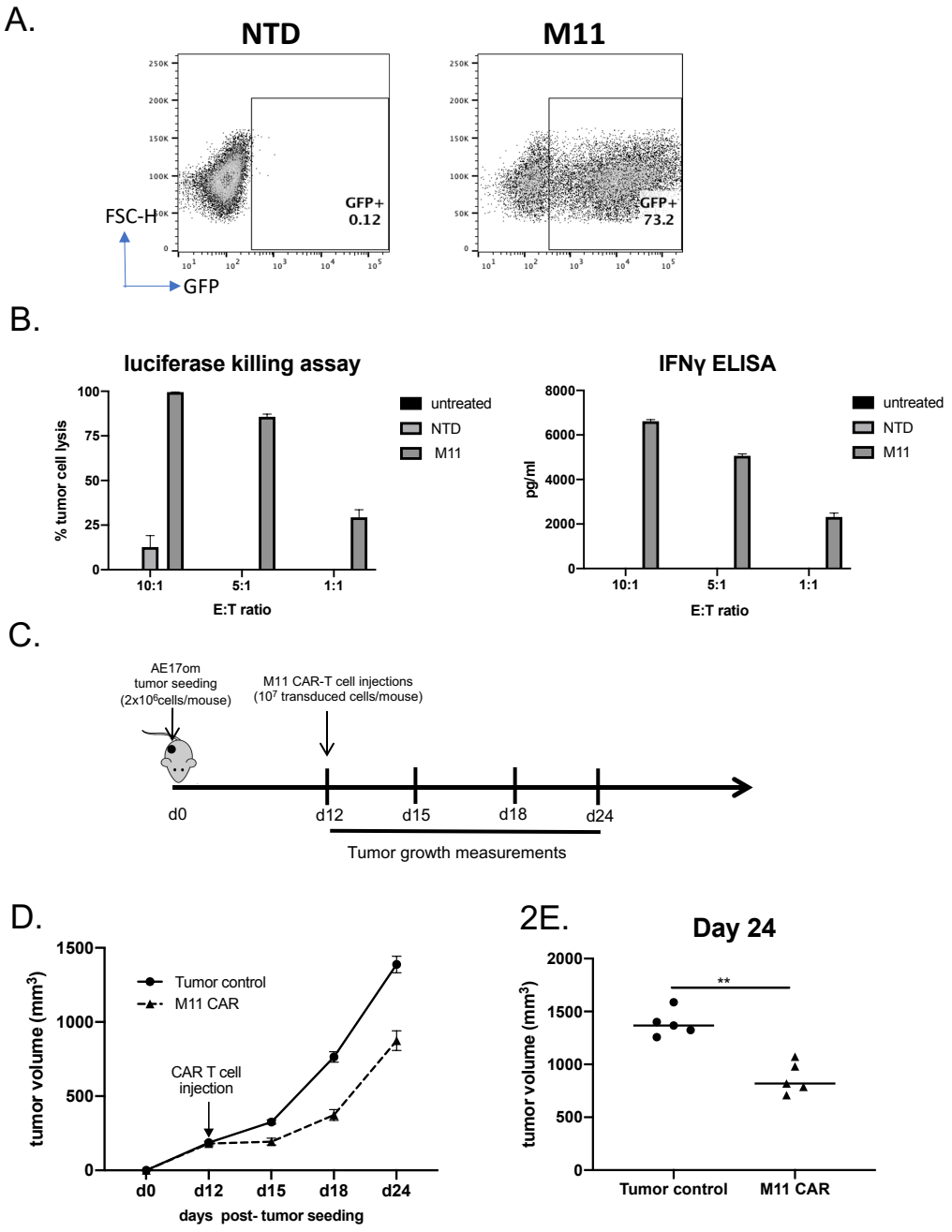


Figure S2. Efficacy of single-dose M11 CAR T cells in the established AE17om MM model. A. Flow tracings assessing the transduction efficiency of M11 CAR T cells prior to administration into the mice **B.** Cytotoxicity of M11 CAR T cells was assessed with a luciferase killing assay **(left graph).** 30,000 tumor cells were seeded in triplicate wells of a 96-well plate and, following adherence after about 4 hrs, no T cells, NTD or M11 CAR T cells were added to wells at E:T ratios of 10:1, 5:1 and 1:1. After overnight incubation, supernatants were collected and cytotoxicity of CAR T cells was measured by killing assay, using the Promega luciferase assay kit. IFNγ production was measured in the co-cultures' supernatants by a mouse IFNγ ELISA kit **(right graph). C.** Schematic of the

experimental plan followed in the in vivo study assessing the effect of M11 CAR T cells in established AE17om MM tumors. $2x10^6$ tumor cells were inoculated in the right flanks of mice. After around 11 days, tumors reached an average size of ~150mm^3. On day 12, a single dose of 10^7 M11 CAR T cells was given intravenously via tail vein injection. Tumor growth was followed via caliper measurements every 2 to 3 days. **D.** Graph of tumor growth in untreated and M11 CAR T cell treated mice, showing that M11 CAR T cells have a minimal effect in slowing tumor growth. Mice n=5 per group. **E.** Bar graph showing the difference in tumor growth between untreated and M11 CAR T cell-treated mice is not significantly significant. Statistics by student t-test.

Figure S

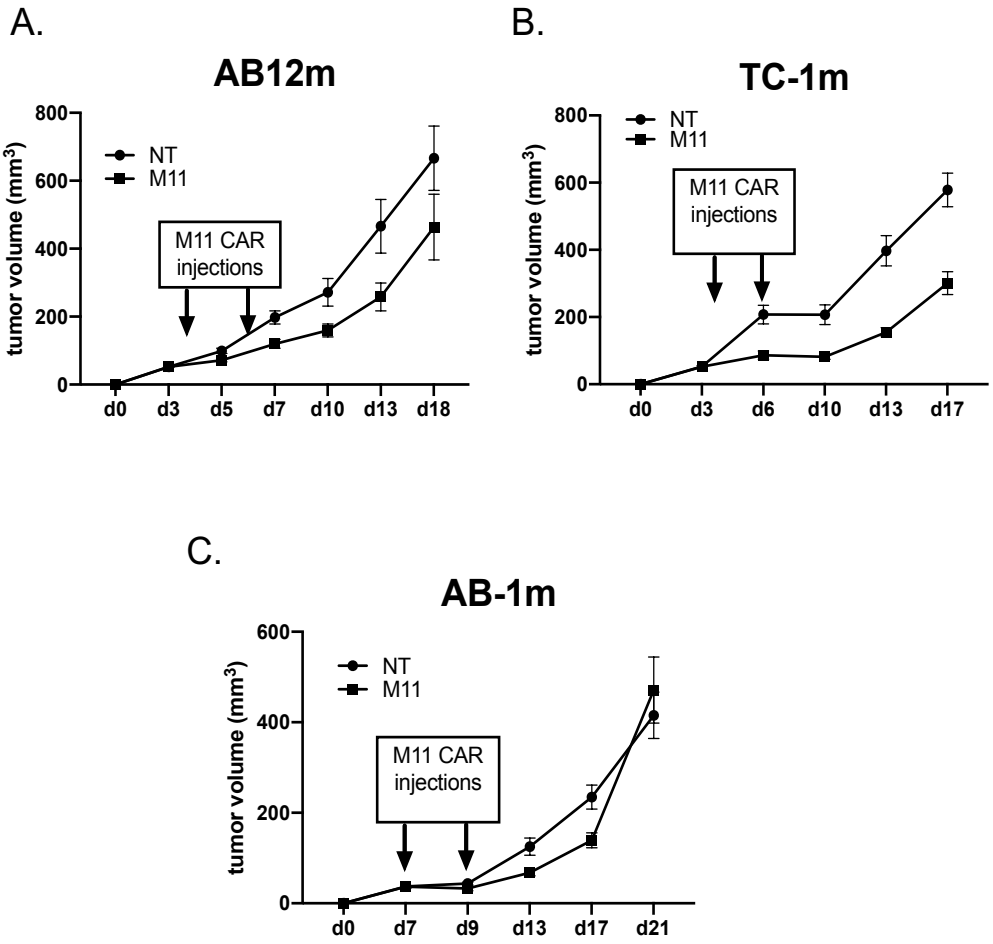


Figure S3. Establishment of other tumor models that can be cured with M11 CAR T cells was unsuccessful. We undertook in vivo experiments with three cell lines, stably transduced with human mesothelin, and tested the ability of M11 CAR T cells to cure established tumors of ~50mm^3, if given in 2 doses of 10^7 cells each, 2 days apart. The treatment showed minimal anti-tumor activity against murine MM AB12m cell line (n= 10 per group) **(A).**

Better efficiency of the M11 CAR T cells was observed in established lung cancer TC-1m tumors, however this did not result in cures (n= 8 per group) **(B).** In another MM in vivo model using AB-1m cells, M11 CAR T cells had no effect in tumor growth (n= 10 per group) **(C).**

Figure S4

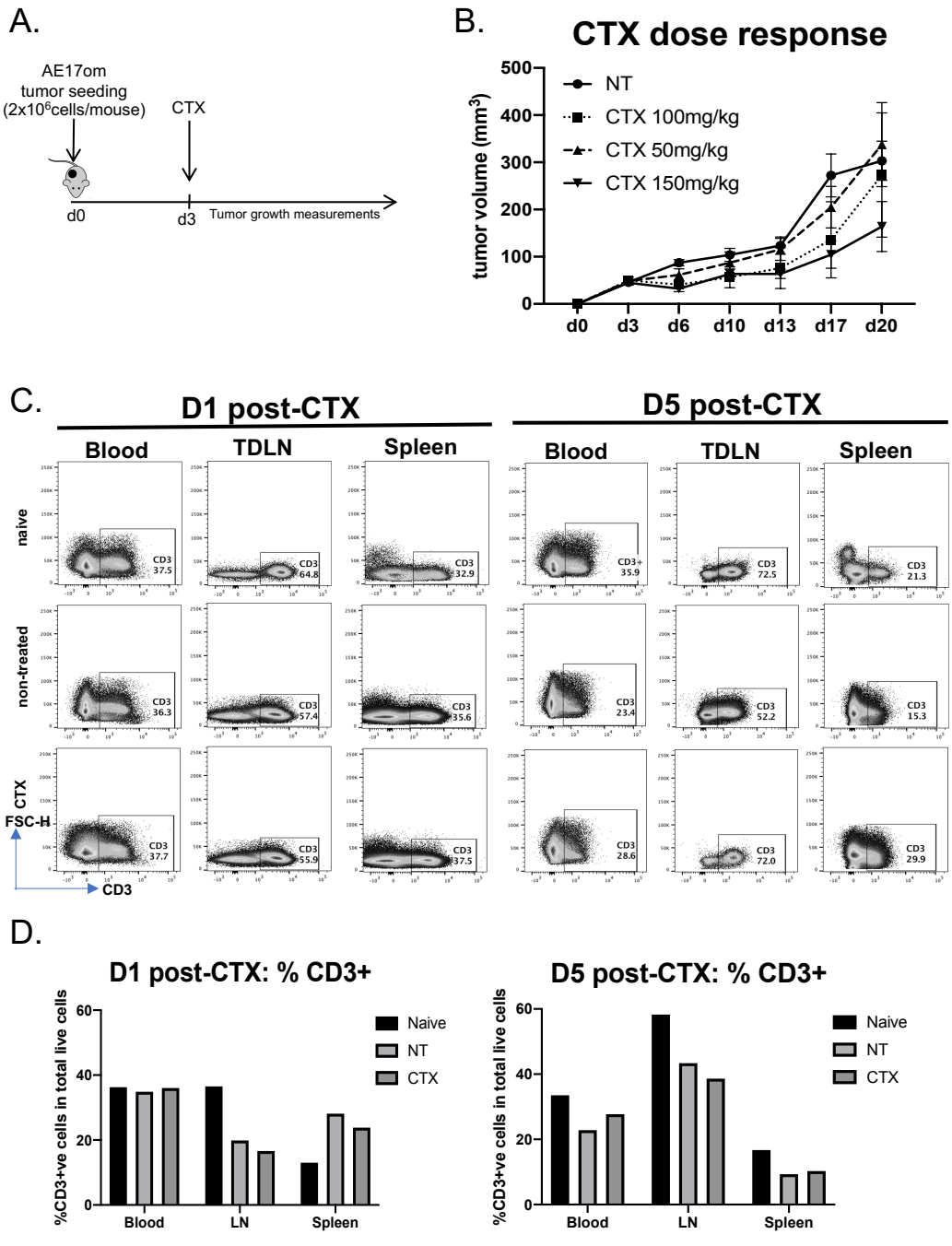


Figure S4. CTX dose-response experiments to determine sub-optimal dose in vivo. A. Schematic of the experimental plan followed to assess CTX cytotoxicity. $2x10^6$ AE17om cells were inoculated in the right flanks of mice. On day 3 post-inoculation, established tumors measured ~$50mm^3$ and treated with a single dose of 50, 100, or 150 mg/kg CTX. Tumor growth was followed for up to 2 weeks' following CTX administration. **B.** Graph from tumor growth measurements over time in mice that were treated with single dose CTX, 3 days post tumor inoculation. A dose response effect of CTX was observed with 50mg/kg CTX having no effect in our tumor model, and a small effect seen with doses of 100 and 150 mg/kg respectively. **C.** Flow tracings assessing lymphodepletion in single cell suspensions from blood, TDLNs and spleens of mice treated with 100 mg/kg CTX on d1 (left panel) and d5 (right panel) post-CTX treatment. CD3+ T lymphocytes were gated on live CD45+ cells. Data is representative of two independent experiments. D. Lymphocyte (CD45+CD3+) frequency in total live cell sample was slightly decreased in the lymph nodes (LN) and spleens, but not in the blood, of CTX-treated mice on d1 post-CTX (left graph). A small decrease in the CD3+ lymphocyte population (of total live cells) was seen in lymph nodes (LN), but not in the blood or spleens, of CTX-treated mice on d5 post-CTX (right graph).

Table S1. Antibodies for Flow cytometric Analysis.

Marker	Clone	Company
CD45	30-F11	Biolegend
CD3	17A2	Biolegend
CD4	GK1.5	Biolegend
CD8	53-6.7	Biolegend
FoxP3	MF-14	Biolegend
CD11c	N4/8	Biolegend
CD64	X54-5/7.1	Biolegend
CD24	M1/69	Biolegend
CD103	2E7	Biolegend
CD11b	M1/70	Biolegend
CLEC9A	7H11	Biolegend
IRF-8	V3GYWCH	Invitrogen
XCR1	ZET	Biolegend
Mesothelin	115-095-072 (code no)	Jackson ImmunoResearch Laboratories, Inc

Supplemental Methods

Cell cultures

All cell lines were cultured and maintained in RPMI (Lonza) supplemented with 10% fetal bovine serum (FBS; Corning), 1% penicillin/streptomycin (Gibco) and 1% L-glutamine (Gibco), at 37ºC/ 5% CO_2. The cell lines were regularly tested and maintained negative for Mycoplasma spp.

T cell isolation, activation, expansion and transduction

Day 0: T cell isolation and activation

1. Harvest spleens in a few ml R10 on ice. Under sterile conditions, decant each spleen onto single-cell 70um filter placed onto a 50ml conical tube. Using the backside of a plunger, mash the spleen up onto the filter membrane surface. Wash the filter with 5ml R10. Add another spleen and continue this process until all spleens are mashed and ~20-30ml are collected in the tube.
2. Centrifuge 1500rpm, 5 min, 4ºC.
3. Aspirate supernatant and add 10ml lysis buffer per spleen (i.e. 50ml for 5 spleens). The lysis buffer is in the cold room- 10x stock, dilute 1:10 in sterile water. Mix tube by tilting a few times.
4. Centrifuge 1500rpm, 5 min, 4ºC. The splenocytes should be pelleted and appear less red.
5. Aspirate the supernatant, add 20ml of R10 into tube and re-suspend the pellet.
6. Filter through a single-cell filter into a new conical tube. Pipette 10ml R10 into the previous tube to wash any remaining cells and pipette this through the filter to a total of 30ml.
7. Take a sample for counting and centrifuge the tube at 1500rpm, 5 min, 4ºC.
8. Isolation of mouse T cells is done using the pan T cell isolation kit and LS columns (Miltenyi Biotech), according to manufacturer's instructions.
9. When cell isolation is complete, prepare the LS column by pipetting ~5ml L15 media into the column, and a waste tube for collection below.
7. After spinning, discard supernatant and re-suspend cells with L15 media to (cell no) x 5ul= 500ul.
8. Drop by drop, passage cell suspension through the column and collect in a new conical tube. Wash column with 3ml L15 media and collect. Repeat.
9. Make up to 10 or 20ml by adding media. Keep some cells for counting and spin the tube.
10. Count mouse CD3/CD28 beads and wash the number of beads you need for a 2:1 bead:Tcell ratio. Wash the beads for 10 mins in PBS against a magnet.
11. After spinning, discard supernatant and resuspend cells in mouse T cell media* containing the beads at 1e6/ml with 50U/ml IL-2 (stock conc. 10,000U/ml).
12. Aliquot 2ml of 1e6/ml cells into 12-well plates, and incubate at 37ºC.

Day 1: T cell expansion

1. Take plate(s) with activated T cells from the incubator and spin at 320xg for 5 min. Remove 0.5 mL of media from each well.
2. Add 0.5mL of mouse T cell media with 50 U/mL of mIL-2 to each well, and then place plates in cell incubator at 37 ^{0}C, 5% CO_2.
3. Coat 24-well non-TC treated plates with 0.5 mL of 20 µg/mL Retronectin (Takara) in 1xPBS at 4 ^{0}C overnight.

Day 2: T cell transduction

1. Remove Retronectin and block with 2% BSA for 20 min at RT.
2. Remove 2% BSA and wash with 1xPBS.
3. Add virus sup at 1 mL per well. Spin plates for 1 hour, 1000xg at RT (acceleration= 0 or slow, deceleration= 0).
4. Collect activated T cells, count, centrifuge at 320xg for 5 min, and resuspend cells at 1E6 cells per mL with 50 U/mL of IL-2.
5. Add 1mL of cells to each well, and then place plates in cell incubator at 37 ^{0}C, 5% CO_2.
6. Cells will be ready to use on ~ Day 5 (3 days post-transduction).

*Mouse T cell media: 1L RPMI containing: 10% FBS, 1% pen/strep, 1mM Pyruvate, 50µM BME.