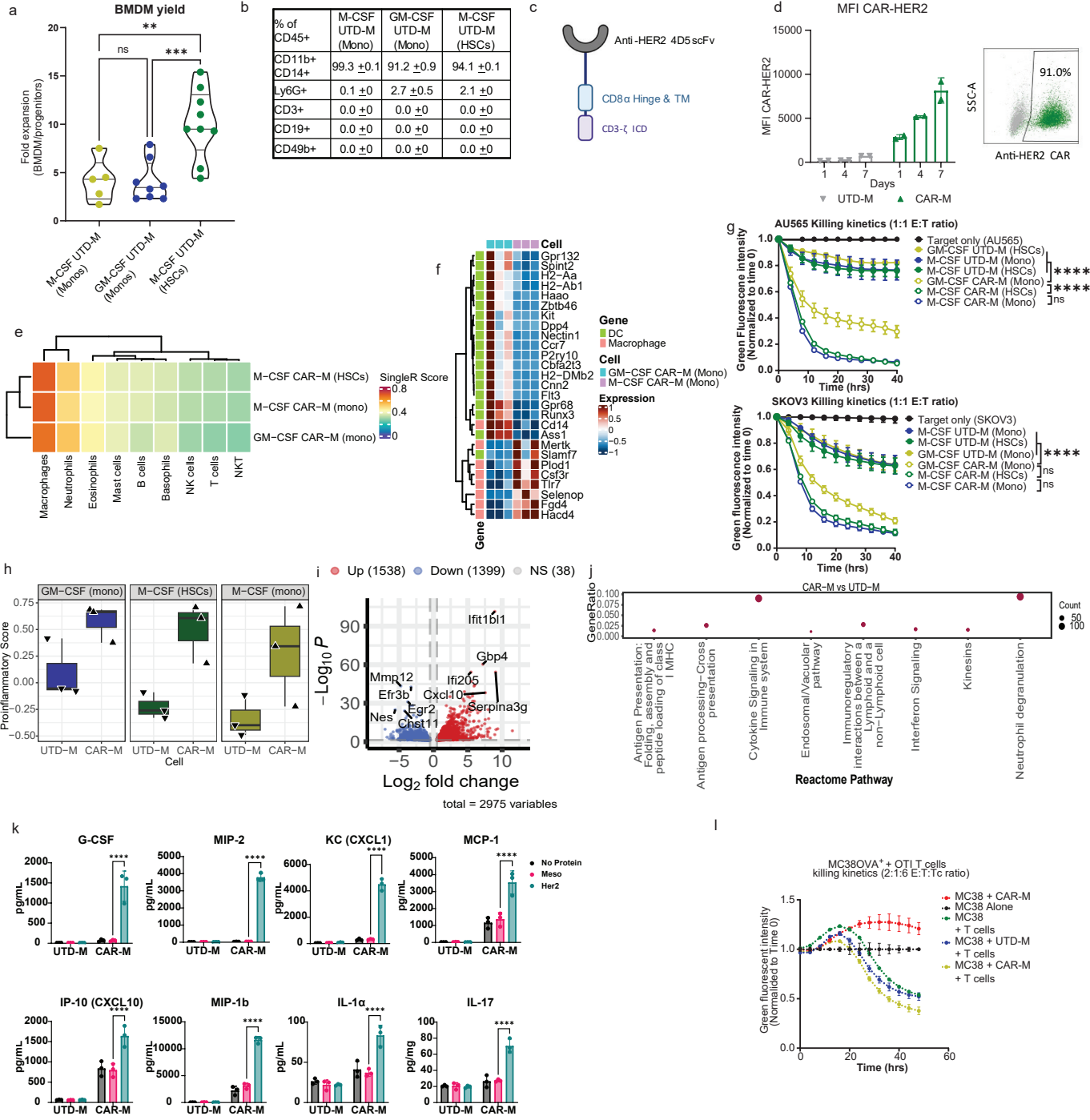
**Extended Table 1. Antibody panel used to stain tumor and immune cells for IONpath multiplex ion beam imaging (MIBI)**

| # | Antibody | Clone | Metal |
|---|---|---|---|
| 1 | dsDNA | 35I9 DNA | 89Y |
| 2 | beta-tubulin | D3U1W | 113In |
| 3 | CD4 | 766 | 143Nd |
| 4 | CD11c | D1V9Y | 144Nd |
| 5 | FOXP3 | FJK-16s | 147Sm |
| 6 | Granzyme B | EPR22645-206 | 150Nd |
| 7 | CD49b | EPR17338 | 151Eu |
| 8 | CD31 | D8V9E | 152Sm |
| 9 | Ki-67 | SP6 | 153Eu |
| 10 | CD11b | EPR1344 | 155Gd |
| 11 | F4/80 | D2S9R | 156Gd |
| 12 | CD8 | CAL38 | 158Gd |
| 13 | CD3e | EPR22667-12 | 159Tb |
| 14 | Vimentin | D21H3 | 163Dy |
| 15 | SMA | D4K9N | 164Dy |
| 16 | PAX5 | D7H5X | 165Ho |
| 17 | Ly6G | 1A8 | 173Yb |
| 18 | CD206 | E2L9N | 174Yb |
| 19 | CD45 | D3F8Q | 175 Lu |
| 20 | Na-K-ATPase | EP1845Y | 176Yb |

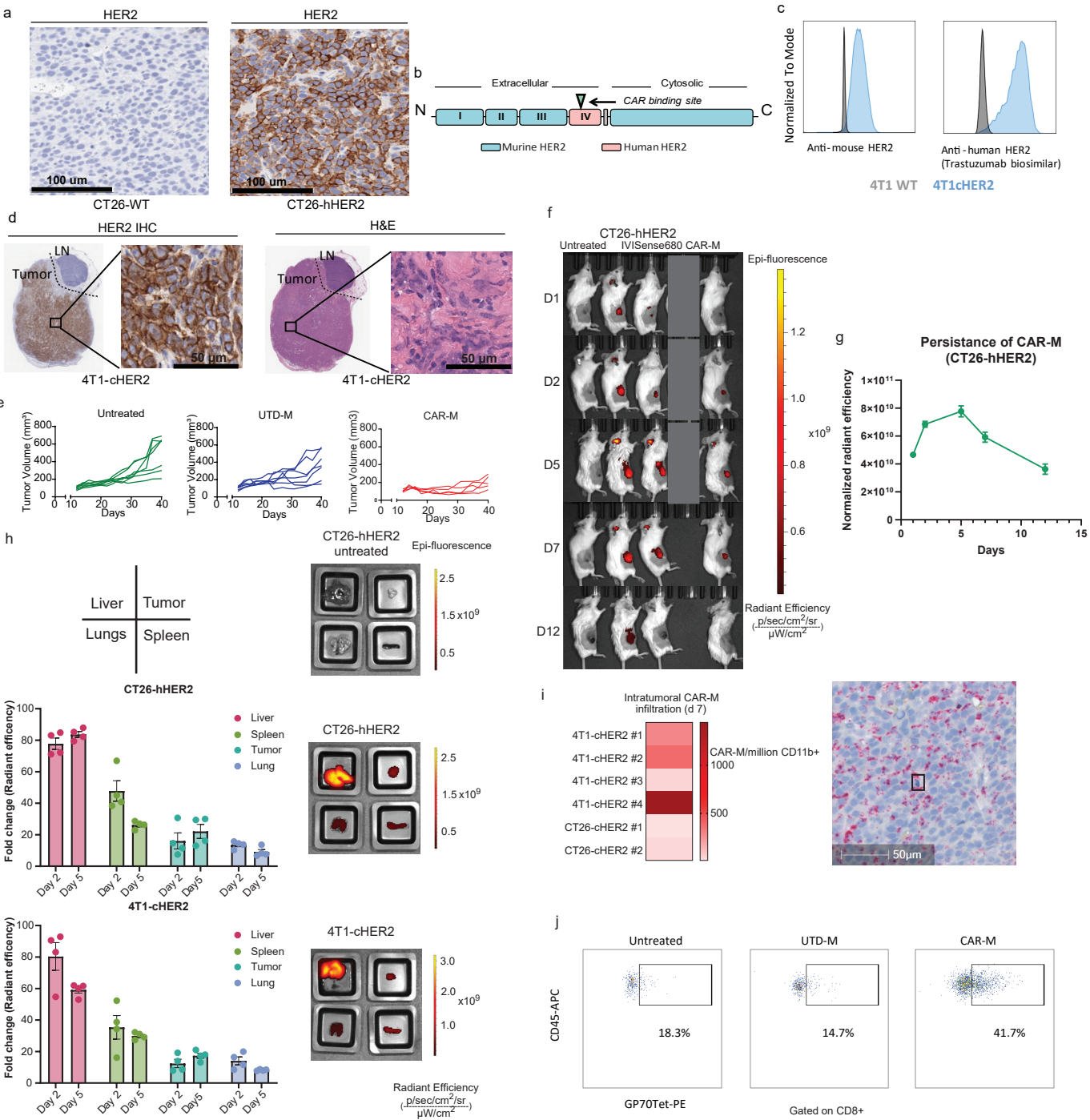
**Extended Table 2. Immune cell populations identified by IONpath multiplex ion beam imaging (MIBI)**

| # | Classified cell populations | Markers |
|---|---|---|
| 1 | Tumor cells & Fibroblast | Vimentin+, SMA- CD45- |
| 2 | Myofibroblasts | Vimentin+, SMA+ |
| 3 | Immune cells | CD45+, CD3+, CD11b+, CD11c+, F4/80+, Ly6G+, PAX5+ |
| 4 | Proliferating immune cells | CD45+, CD3+, CD11b+, CD11c+, F4/80+, Ly6G+, PAX5+, Ki-67+ |
| 5 | T cells | CD45+, CD3+ |
| 6 | Helper T cells | CD45+, CD3+, CD4+, FOXP3- |
| 7 | Activated Helper T cells | CD45+, CD3+, CD4+, FOXP3-, Granzyme B+ |
| 8 | Cytotoxic T cells | CD45+, CD3+, CD8+ |
| 9 | Activated Cytotoxic T cells | CD45+, CD3+, CD8+, Granzyme B+ |
| 10 | Regulatory T cells | CD45+, CD3+, FOXP3+ |
| 11 | CD4+ Regulatory T cells | CD45+, CD3+, CD4+, FOXP3+ |
| 12 | Natural Killer cells | CD45+, CD3-, CD49b+ |
| 13 | Activated Natural Killer cells | CD45+, CD3-, CD49b+, Granzyme B+ |
| 14 | B cells | CD45+, PAX5+ |
| 15 | Macrophages | CD45+, F4/80+ |
| 16 | M1 Macrophages | CD45+, F4/80+, CD206- |
| 17 | M2 Macrophages | CD45+, F4/80+, CD206+ |
| 18 | Dendritc cells | CD45+, CD11c+ |
| 19 | CD8+ Dendritic cells | CD45+, CD11c+, CD8+, CD11b- |
| 20 | CD11b+ Dendritc cells | CD45+, CD11c+, CD8-, CD11b+ |
| 21 | Other myeloid cells | CD45+, CD11b+, CD11c-, F4-80- |
| 22 | Granulocytes/PMN-MDSC | CD45+, CD11b+, CD11c-, F4-80-, Ly6G+ |
| 23 | Monocytes/M-MDSC | CD45+, CD11b+, CD11c-, F4-80-, Ly6G- |


a
BMDM yield
Fold expansion (BMDM/progenitors)
ns
**
***
M-CSF UTD-M (Monos)
GM-CSF UTD-M (Monos)
M-CSF UTD-M (HSCs)
b
% of CD45+
M-CSF UTD-M (Mono)
GM-CSF UTD-M (Mono)
M-CSF UTD-M (HSCs)
CD11b+ CD14+
99.3 ±0.1
91.2 ±0.9
94.1 ±0.1
Ly6G+
0.1 ±0
2.7 ±0.5
2.1 ±0
CD3+
0.0 ±0
0.0 ±0
0.0 ±0
CD19+
0.0 ±0
0.0 ±0
0.0 ±0
CD49b+
0.0 ±0
0.0 ±0
0.0 ±0
c
Anti-HER2 4D5 scFv
CD8α Hinge & TM
CD3-ζ ICD
d
MFI CAR-HER2
MFI CAR-HER2
Days
UTD-M
CAR-M
SSC-A
91.0%
Anti-HER2 CAR
e
M-CSF CAR-M (HSCs)
M-CSF CAR-M (mono)
GM-CSF CAR-M (mono)
SingleR Score
Macrophages
Neutrophils
Eosinophils
Mast cells
B cells
Basophils
NK cells
T cells
NKT
f
Cell
Gene
DC
Macrophage
Cell
GM-CSF CAR-M (Mono)
M-CSF CAR-M (Mono)
Expression
Gpr132
Spint2
H2-Aa
H2-Ab1
Haao
Zbtb46
Kit
Dpp4
Nectin1
Ccr7
P2ry10
Cbfa2t3
H2-DMb2
Cnn2
Flt3
Gpr68
Runx3
Cd14
Ass1
Mertk
Slamf7
Plod1
Csf3r
Tlr7
Selenop
Fgd4
Hacd4
g
AU565 Killing kinetics (1:1 E:T ratio)
Green Fluorescene intensity (Normalized to time 0)
Time (hrs)
Target only (AU565)
GM-CSF UTD-M (HSCs)
M-CSF UTD-M (Mono)
M-CSF UTD-M (HSCs)
GM-CSF CAR-M (Mono)
M-CSF CAR-M (HSCs)
M-CSF CAR-M (Mono)
****
****
ns
SKOV3 Killing kinetics (1:1 E:T ratio)
Green fluorescence intensity (Normalized to time 0)
Time (hrs)
Target only (SKOV3)
M-CSF UTD-M (Mono)
M-CSF UTD-M (HSCs)
GM-CSF UTD-M (Mono)
GM-CSF CAR-M (Mono)
M-CSF CAR-M (HSCs)
M-CSF CAR-M (Mono)
****
ns
ns
h
GM-CSF (mono)
M-CSF (HSCs)
M-CSF (mono)
Proinflammatory Score
UTD-M
CAR-M
Cell
i
Up (1538)
Down (1399)
NS (38)
−Log10 P
Log2 fold change
total = 2975 variables
Ifit1bl1
Gbp4
Mmp12
Ifi205
Efr3b
Cxcl10
Egr2
Serpina3g
Nes
Chst11
j
CAR-M vs UTD-M
GeneRatio
Count
Antigen Presentation: Folding, assembly and peptide loading of class I MHC
Antigen processing−Cross presentation
Cytokine Signaling in Immune system
Endosomal/Vacuolar pathway
Immunoregulatory interactions between a Lymphoid and a non−Lymphoid cell
Interferon Signaling
Kinesins
Neutrophil degranulation
Reactome Pathway
k
G-CSF
MIP-2
KC (CXCL1)
MCP-1
IP-10 (CXCL10)
MIP-1b
IL-1α
IL-17
pg/mL
pg/mg
UTD-M
CAR-M
No Protein
Meso
Her2
****
l
MC38OVA+ + OTI T cells killing kinetics (2:1:6 E:T:Tc ratio)
Green fluorescent intensity (Normalized to Time 0)
Time (hrs)
MC38 + CAR-M + T cells
MC38 Alone + T cells
MC38 + UTD-M + T cells
MC38 + CAR-M + T cells

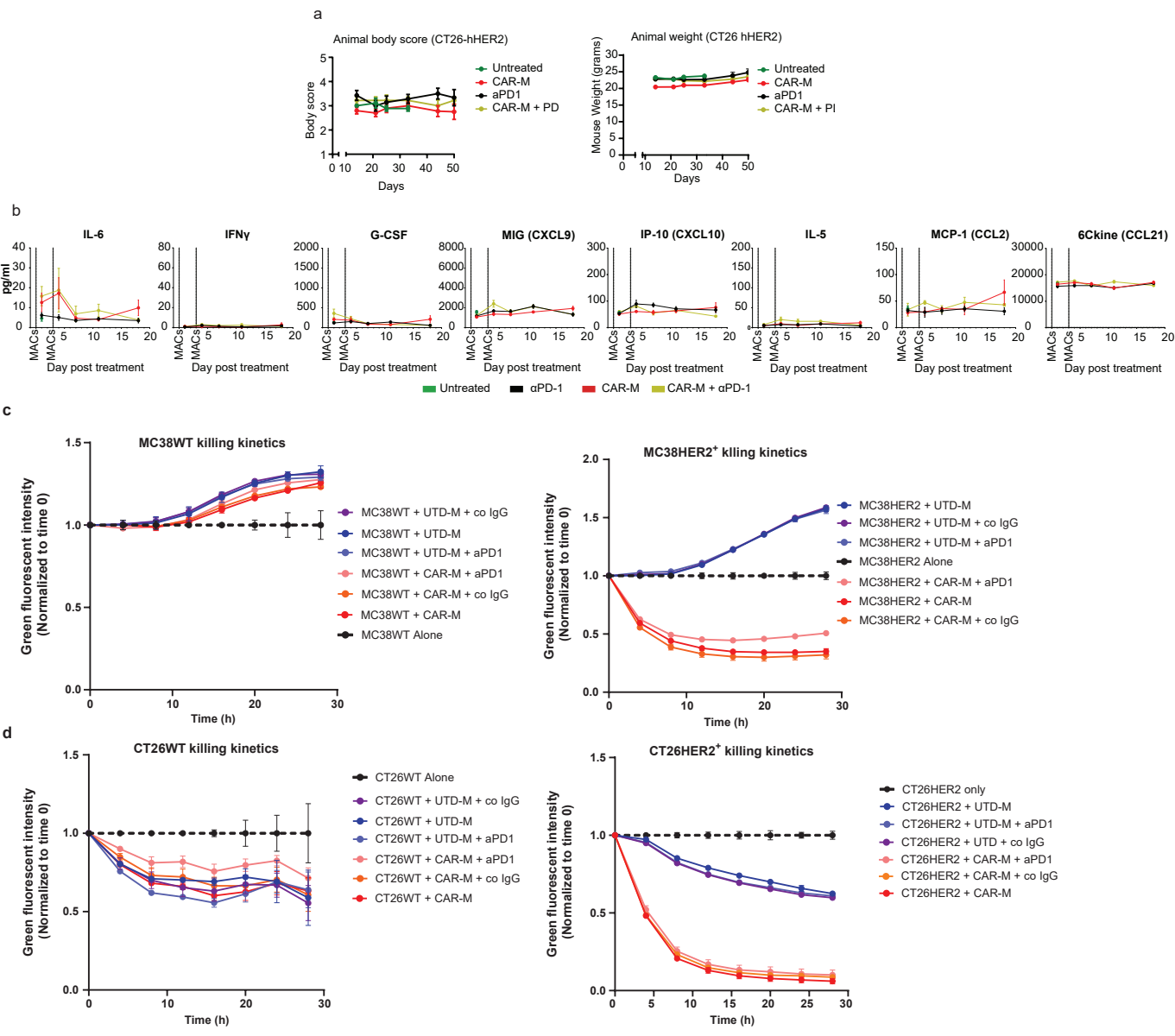
**Supplementary Fig. 1 Generation of murine CAR-Ms and assessment of CAR-mediated tumor cytotoxicity**

**a**, BMDM yield. Data is represented with a truncated violin plot showing all points (*n*=5-9 biological replicate). Statistical analysis was calculated using the ordinary one-way ANOVA. **b**, Purity evaluated by flow cytometry. Data are represented as the mean ± SD of *n*=2 biological replicate. **c**, Diagram of the anti-HER2 CAR construct engineered into the Ad5f35 vector under the control of a CMV promoter. Created in BioRender. Minutolo, N. (2025) https://BioRender.com/i18y106. **d**, Anti-HER2 CAR MFI represented as mean ± SD of *n*=2 technical replicate and representative FACS plot of anti-HER2 CAR expression in M-CSF UTD-M (HSCs). **e**, SingleR prediction analysis assign reference populations to HSC, monocytes, and the three CAR-M cultures (*n*=3 biological replicate). **f**, DESeq2[70] was used to identify significant differentially expressed DC and macrophage genes between GM-CSF CAR-M (mono) and M-CSF CAR-M (mono) (*n*=3 biological replicate). **g**, Incucyte-based killing assays of UTD-M or CAR-M cocultured with SKOV3 NLG or AU565 NLG at E:T, 1:1 for 40 hr. Green fluorescence intensity (GFI) values were normalized to the 0 hr. timepoint and then, further normalized to the target cells. Data is represented as the mean ± SD of *n*=4 technical replicate, and graph is representative of at least three experiments. Ordinary one-way ANOVA was performed at 40 hr. **h,** The pro-inflammatory phenotype was assessed using the proinflammatory score. Data is represented as the mean with Min to Max whiskers of *n*=3 biological replicate. **i**, Volcano plot of M-CSF UTD-M (HSC) vs M-CSF CAR-M (HSC) response gene. Red denotes significantly upregulated genes, and blue indicates significantly downregulated genes (*n*=3 biological replicate). Statistical significance was calculated using DESeq2. **j,** Pathway analysis and gene expression in M-CSF UTD-M (HSC) vs M-CSF CAR-M (HSC) (*n*=3 biological replicate). **k,** CAR-M specificity in response to no protein, mesothelin (Meso; irrelevant antigen control) and HER2. Data are represented as the mean ± SD of *n*=3 biological replicate. Statistical analysis was performed using the two-way ANOVA Tukey's multiple comparisons test. **l**, Killing kinetics of UTD-M and CAR-M co-cultured with MC38OVA$^+$ (HER2 negative) and transgenic CD8$^+$ T cells (OT-1), at E:T:T (Effector:Target:T cells), 2:1:6. GFI values were normalized to the 0 hr. timepoint and then, further normalized to the target cells. Data represents the mean ± SEM of *n*=3 technical replicates and is representative of two experiments. For all images: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Source data are provided as a Source Data file.


a
HER2
HER2
100 um
100 um
CT26-WT
CT26-hHER2
b
Extracellular
Cytosolic
CAR binding site
N
I
II
III
IV
C
Murine HER2
Human HER2
c
Normalized To Mode
Anti-mouse HER2
Anti-human HER2
(Trastuzumab biosimilar)
4T1 WT
4T1cHER2
d
HER2 IHC
H&E
LN
Tumor
50 um
4T1-cHER2
4T1-cHER2
e
Untreated
UTD-M
CAR-M
Tumor Volume (mm3)
Days
f
CT26-hHER2
Untreated
IVISense680 CAR-M
Epi-fluorescence
D1
D2
D5
D7
D12
x10^9
Radiant Efficiency
(p/sec/cm2/sr)/(μW/cm2)
g
Persistance of CAR-M
(CT26-hHER2)
Normalized radiant efficiency
Days
h
Liver
Tumor
Lungs
Spleen
CT26-hHER2
untreated
Epi-fluorescence
CT26-hHER2
CT26-hHER2
Fold change (Radiant efficency)
Liver
Spleen
Tumor
Lung
Day 2
Day 5
4T1-cHER2
4T1-cHER2
Radiant Efficiency
(p/sec/cm2/sr)/(μW/cm2)
i
Intratumoral CAR-M
infiltration (d 7)
4T1-cHER2 #1
4T1-cHER2 #2
4T1-cHER2 #3
4T1-cHER2 #4
CT26-cHER2 #1
CT26-cHER2 #2
CAR-M/million CD11b+
1000
500
50μm
j
Untreated
UTD-M
CAR-M
CD45-APC
18.3%
14.7%
41.7%
GP70Tet-PE
Gated on CD8+

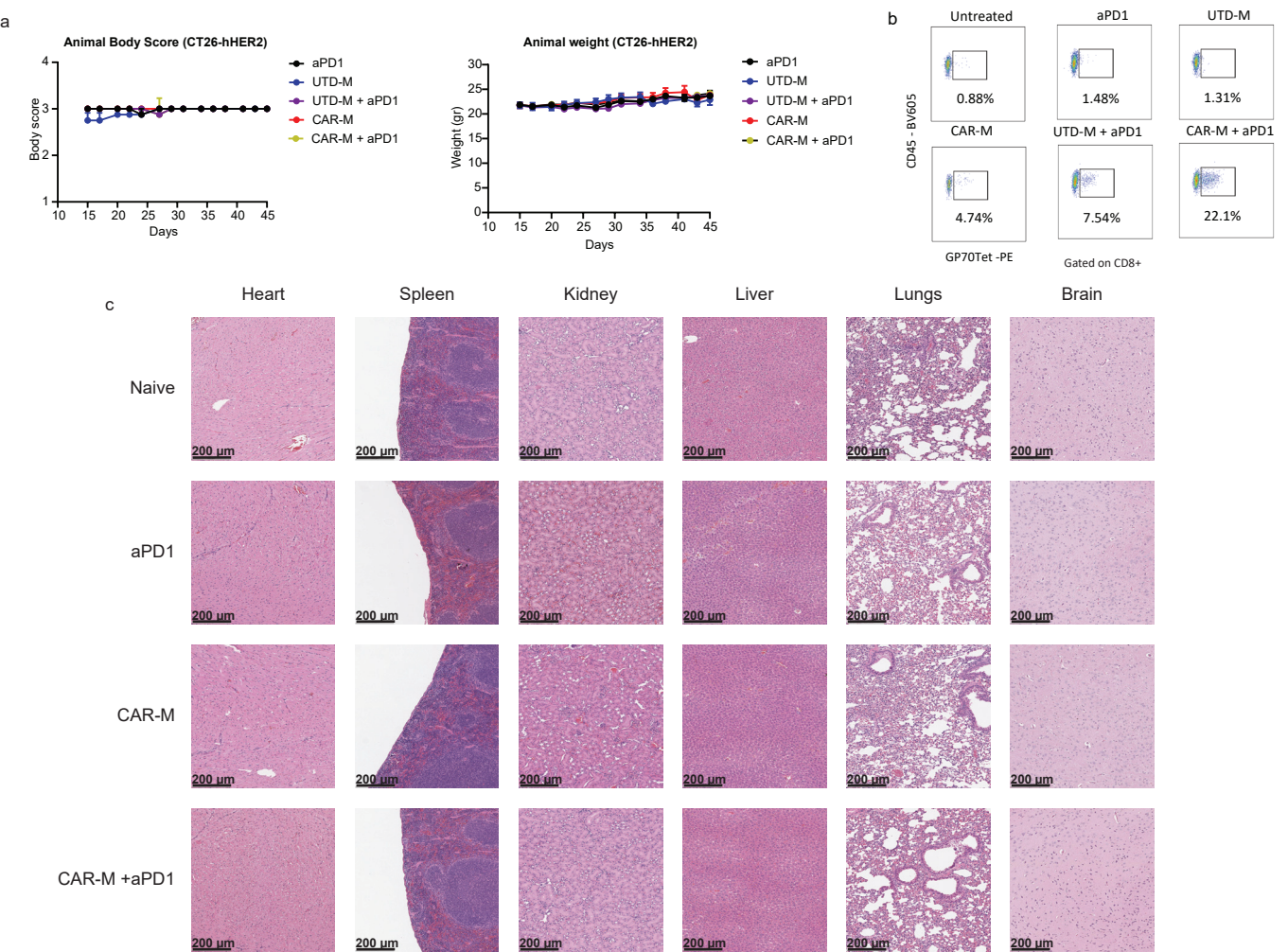
**Supplementary Fig. 2 Generation of the CT26-hHER2 and the 4T1-cHER2 models and assessment of CAR-M persistence and biodistribution**

**a,** IHC analysis on FFPE tumor specimens confirm HER2 expression on CT26-hHER2 implanted subcutaneously in syngeneic BALB/c mice (CT26WT included as a control) evaluated at x20 high power fields per slide. **b,** Diagram of cHER2 construct engineered into a lentiviral vector under the control of CMV promoter. **c,** Representative FACS plots show cHER2 surface expression in 4T1-cHER2 cells upon staining with anti-mouse and anti-human (Trastuzumab biosimilar) antibodies. **d,** IHC analysis on ex vivo FFPE tumor specimen to confirm cHER2 expression on cells orthotopically implanted in the MFP of BALB/c. Lymph nodes (LN) were included as a negative control for HER2 expression and were assessed at x40 high power fields per slide. **e,** 4T1-cHER2 tumor was established orthotopically in the MFP of BALB/c mice for 13 days and treated with IV CAR-M. CAR-M (*n*=5) controlled tumor growth in 4T1-cHER2 compared to untreated (*n*=7) and UTD-M (*n*=7). Tumor growth curves from the individual mouse are shown. **f, g**, $5 \times 10^6$ of fluorescently labeled (IVISense680) murine CAR-M were IV injected into tumor-bearing BALB/c mice. Fluorescence was tracked over a period of 12 days. **f**, IVIS images. **g**, Quantification represented as the mean ± SEM of *n*=3. Values are normalized by subtracting the baseline signal from untreated mouse. **h**, organs and tumors were explanted for ex vivo imaging 2 and 5 days after a single injection of $5 \times 10^6$ CAR-M. Fold change values were calculated as radiant efficiency of treated organ divided by its respective untreated organ. *n*=4 mice per treatment group per tumor type; the experiment was performed once. **i**, Mice with established tumors were treated with three doses of $5 \times 10^6$ CAR-M/mouse every 3-4 days. RNAscope was used to detect CD11b gene (red) and CAR sequence (green) on FFPE sections from tumors collected seven days after the last injection. Heatmap shows the number of CAR-M per $1 \times 10^6$ CD11b (4T1-cHER2 *n*=4 and CT26-hHER2 *n*=2). Representative images at 40x magnification. The square delignates a CAR-M+ event. **j**, Representative FACS plots show % of gp70+ tetramer within intratumoral $CD8^+$ T cells from CT26-hHER2 tumor treated with reginal CAR-M. Source data are provided as a Source Data file.


a
Animal body score (CT26-hHER2)
Body score
Days
Untreated
CAR-M
aPD1
CAR-M + PD
Animal weight (CT26 hHER2)
Mouse Weight (grams)
Days
Untreated
CAR-M
aPD1
CAR-M + PI
b
IL-6
IFNγ
G-CSF
MIG (CXCL9)
IP-10 (CXCL10)
IL-5
MCP-1 (CCL2)
6Ckine (CCL21)
pg/ml
MACs
Day post treatment
Untreated
αPD-1
CAR-M
CAR-M + αPD-1
c
MC38WT killing kinetics
Green fluorescent intensity (Normalized to time 0)
Time (h)
MC38WT + UTD-M + co IgG
MC38WT + UTD-M
MC38WT + UTD-M + aPD1
MC38WT + CAR-M + aPD1
MC38WT + CAR-M + co IgG
MC38WT + CAR-M
MC38WT Alone
MC38HER2+ kling kinetics
Green fluorescent intensity (Normalized to time 0)
Time (h)
MC38HER2 + UTD-M
MC38HER2 + UTD-M + co IgG
MC38HER2 + UTD-M + aPD1
MC38HER2 Alone
MC38HER2 + CAR-M + aPD1
MC38HER2 + CAR-M
MC38HER2 + CAR-M + co IgG
d
CT26WT killing kinetics
Green fluorescent intensity (Normalized to time 0)
Time (h)
CT26WT Alone
CT26WT + UTD-M + co IgG
CT26WT + UTD-M
CT26WT + UTD-M + aPD1
CT26WT + CAR-M + aPD1
CT26WT + CAR-M + co IgG
CT26WT + CAR-M
CT26HER2+ killing kinetics
Green fluorescent intensity (Normalized to time 0)
Time (h)
CT26HER2 only
CT26HER2 + UTD-M
CT26HER2 + UTD-M + aPD1
CT26HER2 + UTD + co IgG
CT26HER2 + CAR-M + aPD1
CT26HER2 + CAR-M + co IgG
CT26HER2 + CAR-M

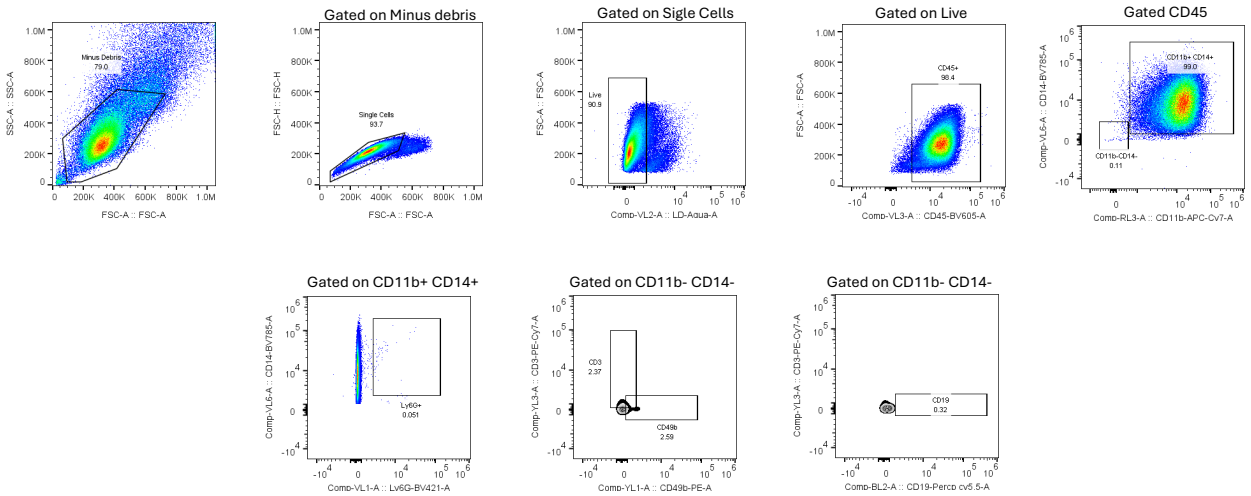
**Supplementary Fig. 3. Regional CAR-M + aPD1 combination therapy preserves body condition score, body weight, and serum cytokines/chemokines levels**

CT26-hHER2 tumor was established SQ in BALB/c and treated with IT CAR-M starting at day 12 and IP PD1 from day 12 until day 36 (approximately every 3 days). **a,** Body condition score and body weight were evaluated in CAR-M (*n*=10), anti-PD1 (*n*=7), CAR-M + aPD1 (*n*=9), or untreated (*n*=9) groups. Data represents the mean ± SEM. **b,** Serum IL-6, IFNγ, G-CSF, MIG (CXCL9), IP-10 (CXCL10), IL-5, MCP-1 (CCL2) and 6Ckine (CCL21) levels were quantified by multiplexed Luminex assay up to 18 days post treatment. Dotted lines represent CAR-M doses. Data represents the mean ± SEM of *n*=6-7/group and is representative of two experiments. **c,d,** Incucyte-based killing assays of UTD-M or CAR-M co-cultured with either MC38 or CT26, at E:T ratio 2:1 in the presence 10 μg/mL IgG control or 10 μg/mL aPD1. Green fluorescence intensity (GFI) values were normalized to the 0 hr. timepoint and then, further normalized to the target cells. Data represents the mean ± SEM of *n*=3 technical replicate and is representative of two experiments. Source data are provided as a Source Data file.

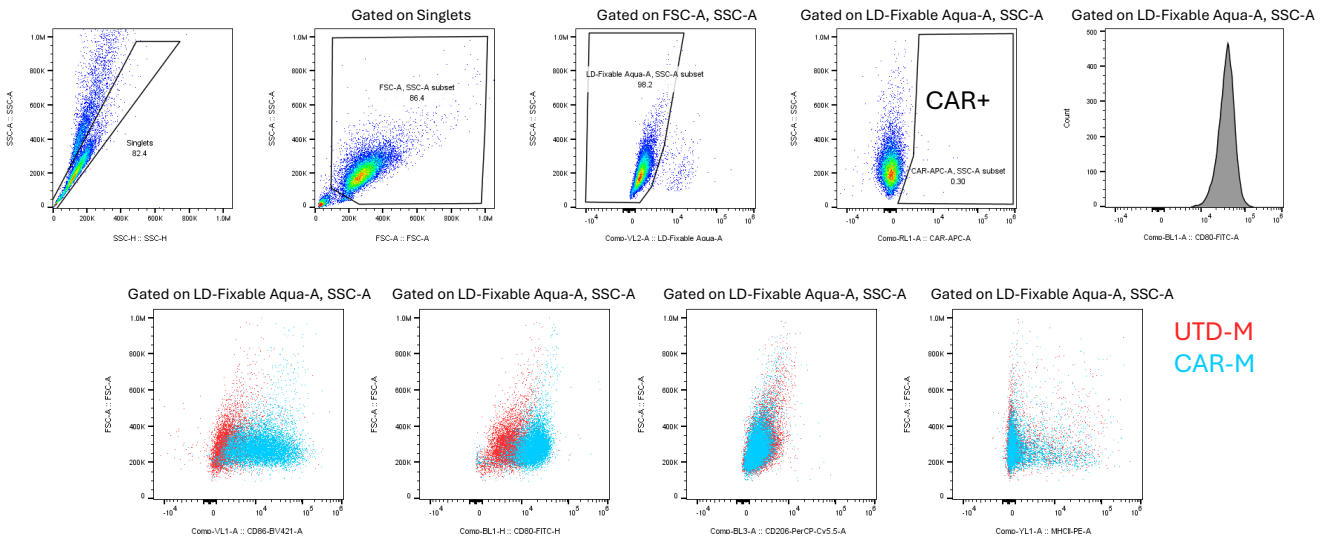

a
Animal Body Score (CT26-hHER2)
Body score
Days
aPD1
UTD-M
UTD-M + aPD1
CAR-M
CAR-M + aPD1
Animal weight (CT26-hHER2)
Weight (gr)
Days
b
Untreated
aPD1
UTD-M
CD45 - BV605
0.88%
1.48%
1.31%
CAR-M
UTD-M + aPD1
CAR-M + aPD1
4.74%
7.54%
22.1%
GP70Tet -PE
Gated on CD8+
c
Heart
Spleen
Kidney
Liver
Lungs
Brain
Naive
aPD1
CAR-M
CAR-M +aPD1
200 µm

**Supplementary Fig. 4. Systemic CAR-M and aPD-1 combination therapy is well tolerated**

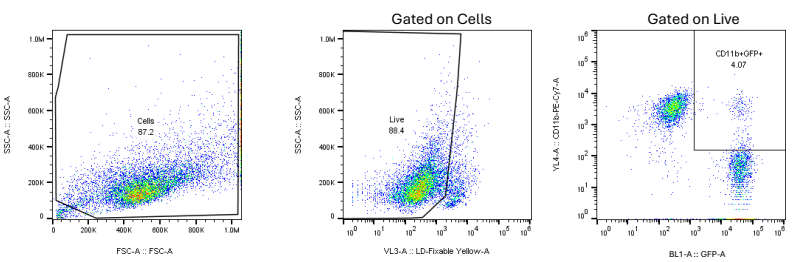
CT26-hHER2 tumor-bearing mice were treated with systemic IV CAR-M from day 10 and IP aPD1 from day 14. **a,** Body condition score and body weight are maintained in response to CAR-M + aPD1 combination therapy. Data represents the mean ± SEM of *n*=8/group except UTD-M + aPD1 *n*=7 and is representative of three experiments. **b**, Representative FACS plots show % of gp70+ tetramer within intratumoral $CD8^+$ T cells from CT26-hHER2 tumor treated with systemic CAR-M. **c**, Histological assessment of heart, spleen, kidney, liver, lung, and brain harvested from CT26-hHER2 tumor-bearing BALB/c mice treated with systemic CAR-M (*n*=4/group). Organs from tumor-free (naïve) mice were included as controls *n*=4. No signs of treatment-related toxicity were observed. Source data are provided as a Source Data file.



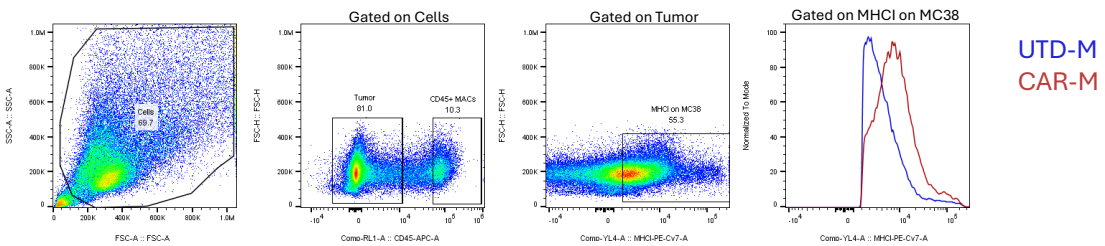

Supplementary Figure 5a



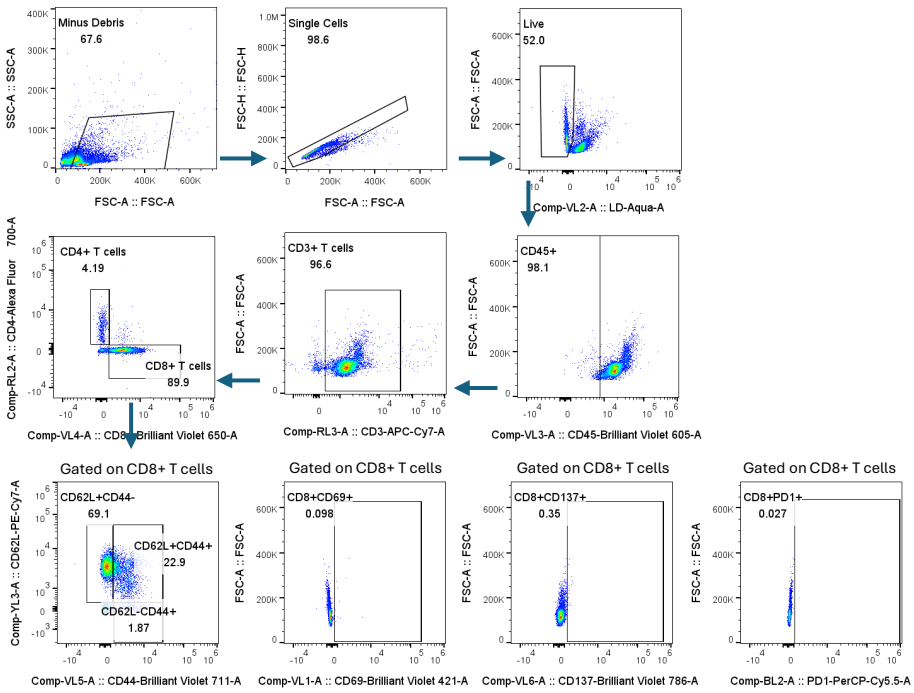

Supplementary Figure 5b



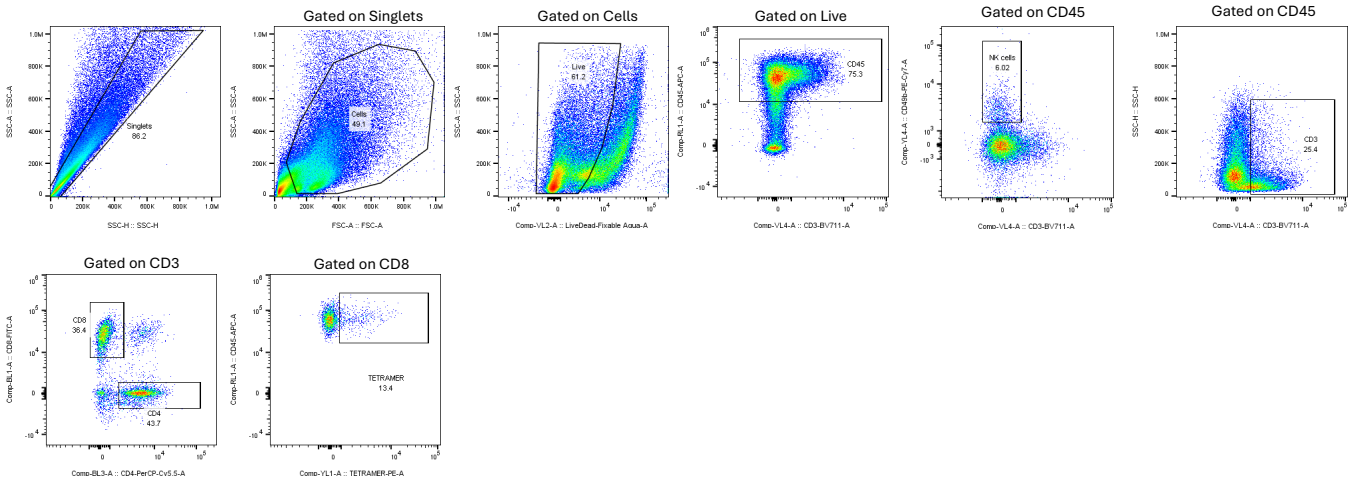

Supplementary Figure 5c



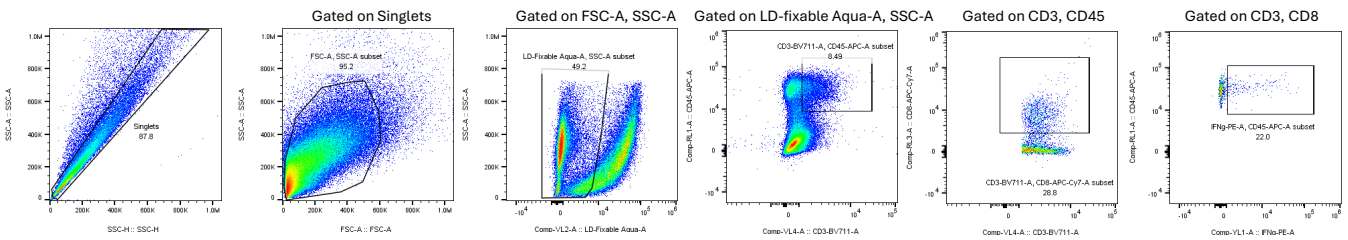

Supplementary Figure 5d



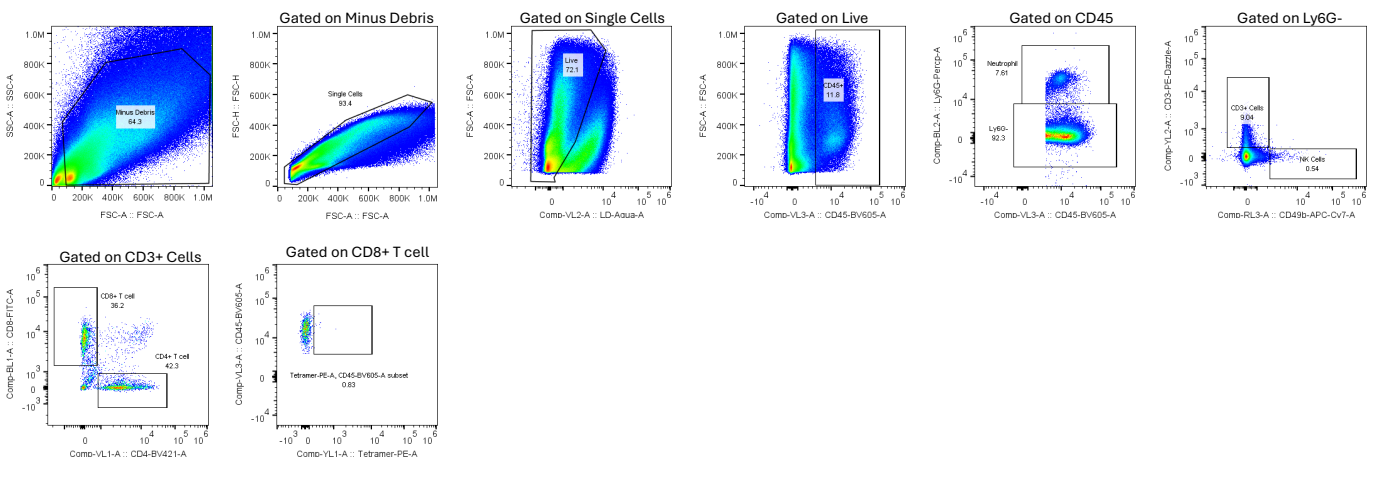

Supplementary Figure 6a




Supplementary Figure 6b




Supplementary Figure 6c




Supplementary Figure 6d