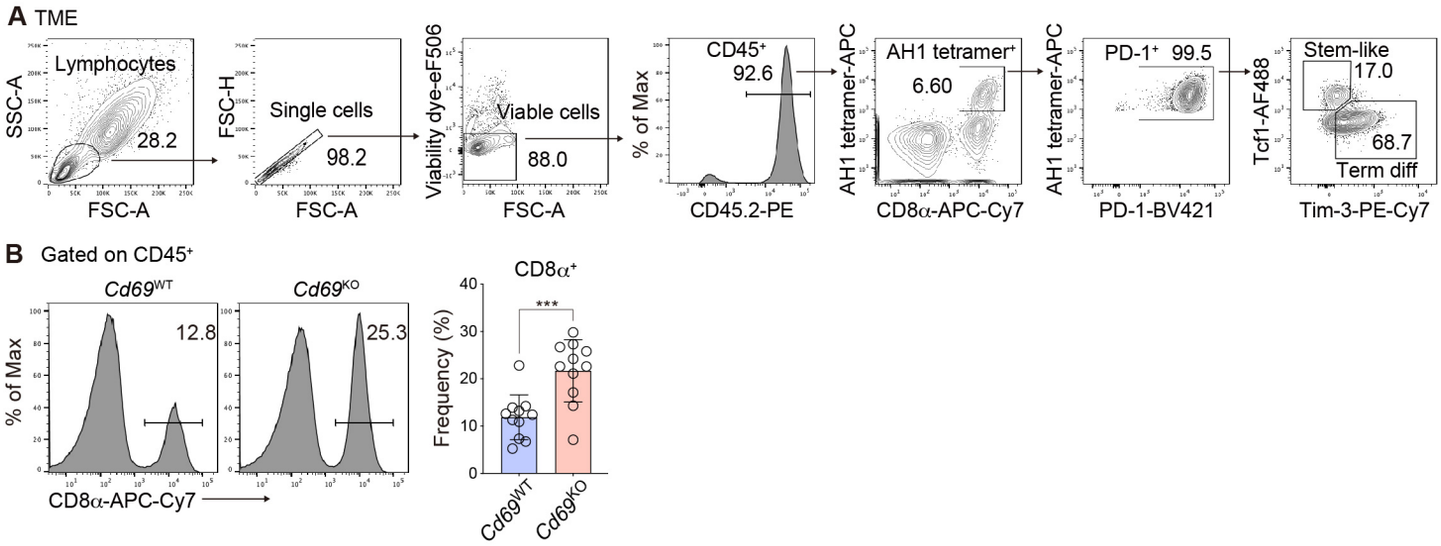
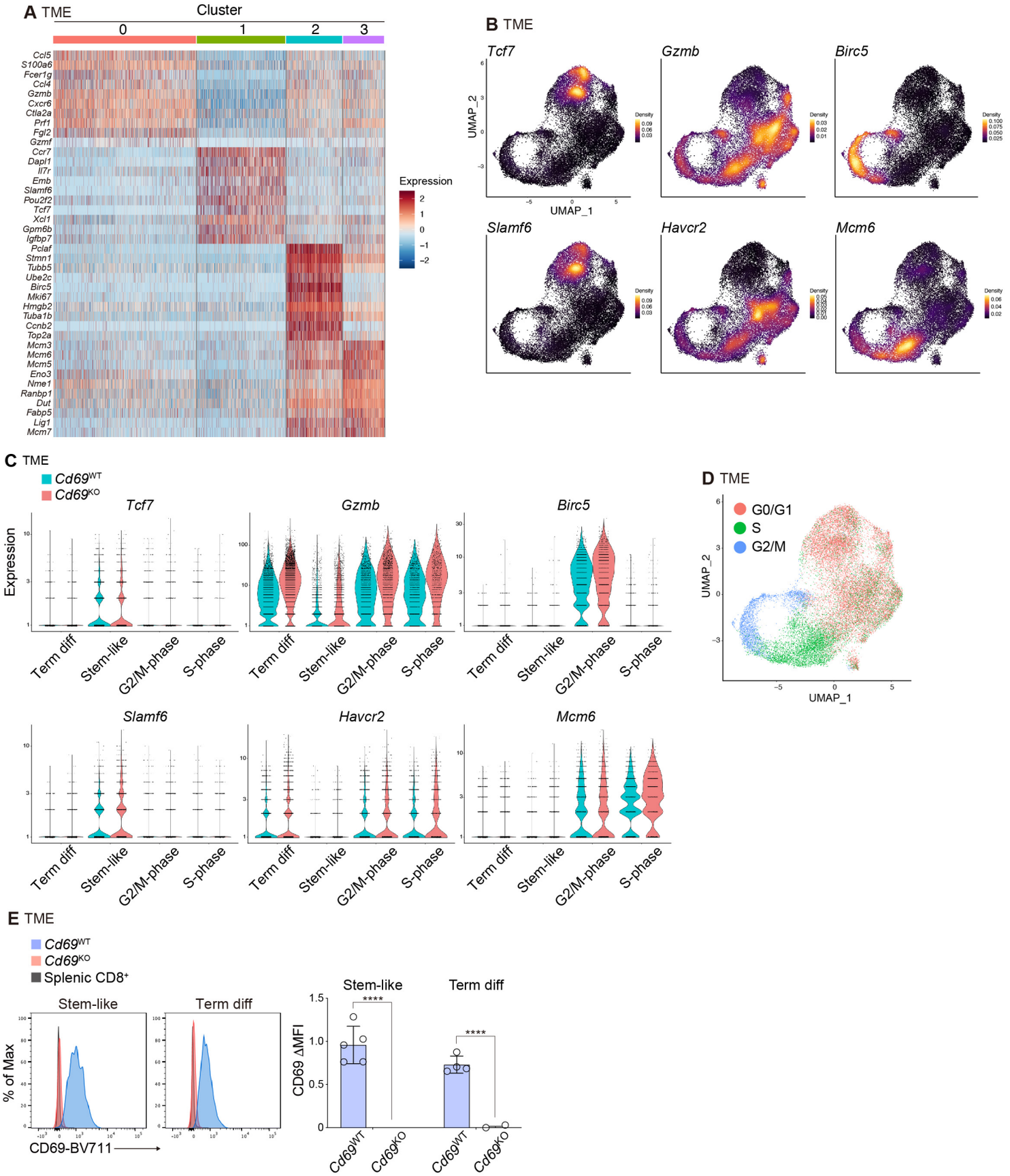
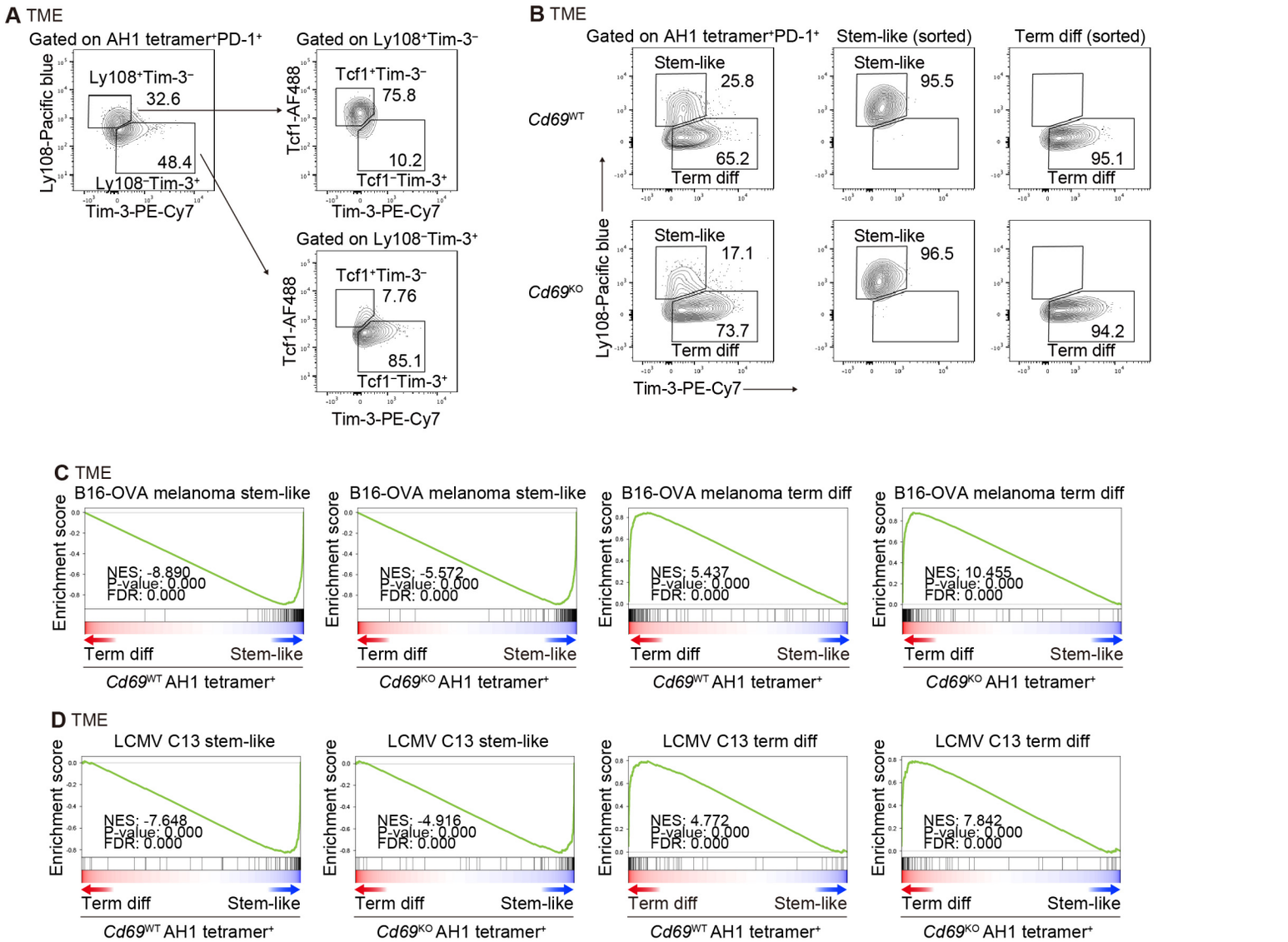

A TME
Lymphocytes
28.2
SSC-A
FSC-A
Single cells
98.2
FSC-H
FSC-A
Viable cells
88.0
Viability dye-eF506
FSC-A
CD45+
92.6
% of Max
CD45.2-PE
AH1 tetramer+
6.60
AH1 tetramer-APC
CD8α-APC-Cy7
PD-1+ 99.5
AH1 tetramer-APC
PD-1-BV421
Stem-like
17.0
68.7
Term diff
Tcf1-AF488
Tim-3-PE-Cy7
B Gated on CD45+
Cd69WT
Cd69KO
12.8
25.3
% of Max
CD8α-APC-Cy7
CD8α+
***
Frequency (%)
Cd69WT
Cd69KO

Supplementary Figure S1. Flow cytometry of intratumoral tumor-specific $CD8^+$ T cells. **A**, A representative flow cytometry gating strategy for intratumoral $CD8^+$ T cell subpopulations. CT26 cells were subcutaneously inoculated into 7-week-old female BALB/c mice. Intratumoral AH1 $tetramer^+$ $CD8^+$ T cells were analyzed at 14 days after inoculation. **B**, CT26 cells were subcutaneously inoculated into *Cd69*^{WT} and *Cd69*^{KO} 7-week-old female BALB/c mice. Intratumoral $CD8^+$ T cells were analyzed at 14 days after CT26 cell inoculation. At least n=6 per group. The number indicates the percentage of gated cells. The frequency of $CD8^+$ T cells in $CD45^+$ cells is shown in representative flow plots (left) and a summary of the experiments (right). An unpaired two-sided Student's *t*-test or Welch's *t*-test was used for the analysis of the flow cytometry data. *** $P<0.001$. Data are pooled from two experiments with at least five mice (**B**).


A TME
Cluster
0
1
2
3
Ccl5
S100a6
Fcer1g
Ccl4
Gzmb
Cxcr6
Ctla2a
Prf1
Fgl2
Gzmf
Ccr7
Dapl1
Il7r
Emb
Slamf6
Pou2f2
Tcf7
Xcl1
Gpm6b
Igfbp7
Pclaf
Stmn1
Tubb5
Ube2c
Birc5
Mki67
Hmgb2
Tuba1b
Ccnb2
Top2a
Mcm3
Mcm6
Mcm5
Eno3
Nme1
Ranbp1
Dut
Fabp5
Lig1
Mcm7
Expression
2
1
0
−1
−2
B TME
Tcf7
Gzmb
Birc5
Slamf6
Havcr2
Mcm6
UMAP_2
UMAP_1
Density
C TME
Cd69WT
Cd69KO
Tcf7
Gzmb
Birc5
Slamf6
Havcr2
Mcm6
Expression
Term diff
Stem-like
G2/M-phase
S-phase
D TME
G0/G1
S
G2/M
UMAP_2
UMAP_1
E TME
Cd69WT
Cd69KO
Splenic CD8+
Stem-like
Term diff
% of Max
CD69-BV711
CD69 ΔMFI
****
****
Cd69WT
Cd69KO
Cd69WT
Cd69KO

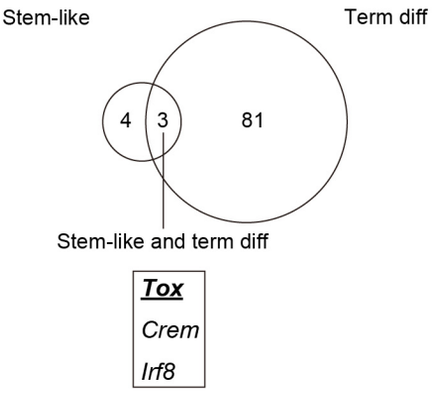
Supplementary Figure S2. scRNA-seq of intratumoral tumor-specific $CD8^+$ T cells. **A**, scRNA-seq. CT26 cells were subcutaneously inoculated into *Cd69*^{WT} and *Cd69*^{KO} 7-week-old female BALB/c mice. Intratumoral AH1 tetramer$^+$ $CD8^+$ T cells were isolated at 14 days after inoculation. Experiments were performed in duplicate. Pool of four mice in 1 group. A UMAP projection of *Cd69*^{WT} (n=9,870 and 4,994 cells) and *Cd69*^{KO} (n=6,090 and 7,551 cells) cells that were assigned to 4 clusters and color-coded based on the clusters, determined by Seurat. A heatmap of the top 10 genes expressed in each cluster as defined in Fig. 1F. The columns correspond to the cells, the rows correspond to the genes. Cells are grouped by clusters. The color scale is based on a z-score distribution from -2 (blue) to 2 (red). **B**, Single-cell transcript levels of the indicated genes illustrated in the UMAP plots. **C**, Violin plots showing the expression of the indicated genes in cells from each cluster as defined in Fig. 1F. The violin represents the probability density at each value. Each dot represents a single cell. **D**, The cell-cycle phase (G0/G1, S, or G2/M) of each cell was determined by Seurat and is shown in the UMAP plots. **E**, CT26 cells were subcutaneously inoculated into *Cd69*^{WT} and *Cd69*^{KO} 7-week-old female BALB/c mice. Intratumoral AH1 tetramer$^+$ $CD8^+$ T cells were analyzed at 14 days after inoculation. At least n=4 per group. Delta MFI of CD69 (delta MFI was calculated by subtracting splenic $CD8^+$ MFI from AH1-specific $CD8^+$ T MFI in $CD8^+$ T cell subpopulations). A representative histogram (left) and summary of the experiments (right). An unpaired two-sided Student's *t*-test or Welch's *t*-test was used for the analysis of the flow cytometry data. **** $P<0.0001$. ns: not significant. Representative data from three experiments with at least four mice are shown (**E**).


A TME
Gated on AH1 tetramer+PD-1+
Ly108+Tim-3−
32.6
48.4
Ly108−Tim-3+
Ly108-Pacific blue
Tim-3-PE-Cy7
Gated on Ly108+Tim-3−
Tcf1+Tim-3−
75.8
10.2
Tcf1−Tim-3+
Tcf1-AF488
Gated on Ly108−Tim-3+
Tcf1+Tim-3−
7.76
85.1
Tcf1−Tim-3+
B TME
Gated on AH1 tetramer+PD-1+
Stem-like (sorted)
Term diff (sorted)
Cd69WT
Cd69KO
Stem-like
25.8
65.2
Term diff
95.5
95.1
17.1
73.7
96.5
94.2
C TME
B16-OVA melanoma stem-like
B16-OVA melanoma term diff
Enrichment score
NES: -8.890
P-value: 0.000
FDR: 0.000
NES: -5.572
NES: 5.437
NES: 10.455
Term diff
Stem-like
Cd69WT AH1 tetramer+
Cd69KO AH1 tetramer+
D TME
LCMV C13 stem-like
LCMV C13 term diff
NES: -7.648
NES: -4.916
NES: 4.772
NES: 7.842

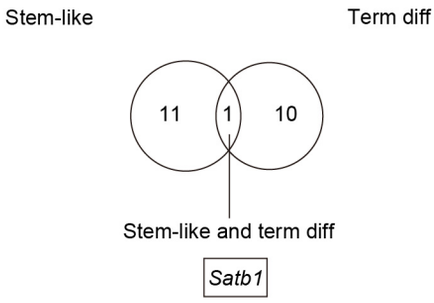
Supplementary Figure S3. Bulk RNA-seq of intratumoral tumor-specific $CD8^+$ T cells. **A**, CT26 cells were subcutaneously inoculated into *Cd69*^{WT} and *Cd69*^{KO} 7-week-old female BALB/c mice. Fourteen days after inoculation, the intratumoral AH1 tetramer$^+$ $CD8^+$ T cell subpopulations were analyzed. For live cell sorting, the cell surface marker Ly108 was used instead of Tcf1 (1). **B**, The intratumoral AH1 tetramer$^+$ $CD8^+$ T cell subpopulations were sorted and subjected to RNA-seq. Experiments were performed in duplicate. Pool of four mice in 1 group. A representative flow cytometry gating strategy for Ly108$^+$ stem-like and Tim-3$^+$ terminally differentiated $CD8^+$ T cells is shown. The number indicates the percentage of gated cells. **C**, A GSEA of the transcriptional signature from stem-like or terminally differentiated $CD8^+$ T cells in B16-OVA melanoma in the ranked list of genes differentially expressed by AH1 tetramer$^+$ terminally differentiated $CD8^+$ T cells versus stem-like $CD8^+$ T cells in *Cd69*^{WT} and *Cd69*^{KO} mice. **D**, A GSEA of the transcriptional signature from stem-like or terminally differentiated $CD8^+$ T cells in LCMV clone-13 infection in the ranked list of genes differentially expressed by AH1 tetramer$^+$ terminally differentiated $CD8^+$ T cells versus stem-like $CD8^+$ T cells in *Cd69*^{WT} and *Cd69*^{KO} mice. The gene expression profiles of $CD8^+$ T cell subpopulations in B16-OVA melanoma and LCMV clone-13 infection were obtained from Miller et al. (1).

A TME

Downregulated transcription factors in *Cd69*^{KO}

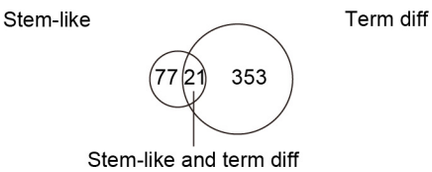


Upregulated transcription factors in *Cd69*^{KO}



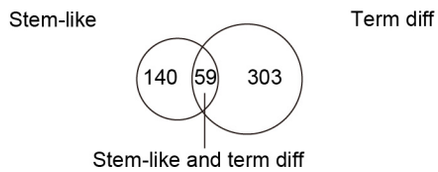
B TME

Downregulated genes in *Cd69*^{KO}



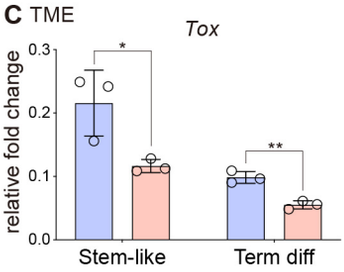
Actn1	*Ncf1*
Cd69	*Oaz1.ps*
Cops7a	*Pcyt1a*
Crem	*Phpt1*
Ddit4	*Pkp4*
Defa4	*Sft2d2*
Hspa1l	*Slc16a11*
Irf8	*Spry2*
Itga4	*Tox*
Klrc3	
Mmd	
Mrc2	

Upregulated genes in *Cd69*^{KO}

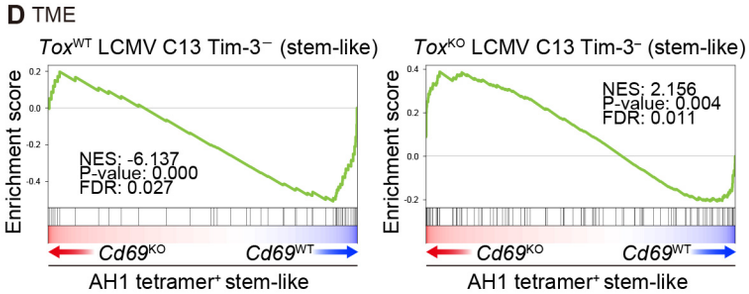


BC021614	*Fam189b*	*Hist1h2be*	*Klrc2*	*Penk*
Casp1	*Fgl2*	*Hist1h2bg*	*Klrd1*	*S100a11*
Casp4	*Flot1*	*Hist1h2bh*	*Krtcap2*	*S100a6*
Ccl3	*Gabarapl1*	*Hist1h4b*	*Lgals3*	*Sar1b*
Cd2	*Glrx*	*Hist1h4h*	*Ly6a*	*Satb1*
Cd8b1	*Gpr68*	*Hist2h2ab*	*Ly6c1*	*Sh3bgrl3*
Cdca3	*Gzmb*	*Hist2h2ac*	*Ly6c2*	*Spp1*
Csf1	*Higd2a*	*Hist2h3c2*	*March3*	*Susd3*
Ctla2a	*Hist1h2aa*	*Hist2h4*	*Mrps16*	*Tmem120a*
Ctla2b	*Hist1h2ak*	*Ier3*	*Ms4a4c*	*Ufc1*
Dpm3	*Hist1h2ba*	*Ifitm3*	*Nop10*	*Vim*
Erdr1	*Hist1h2bc*	*Klrc1*	*Orai2*	

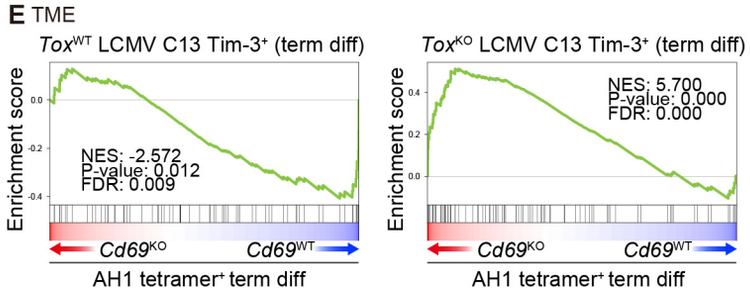
C TME



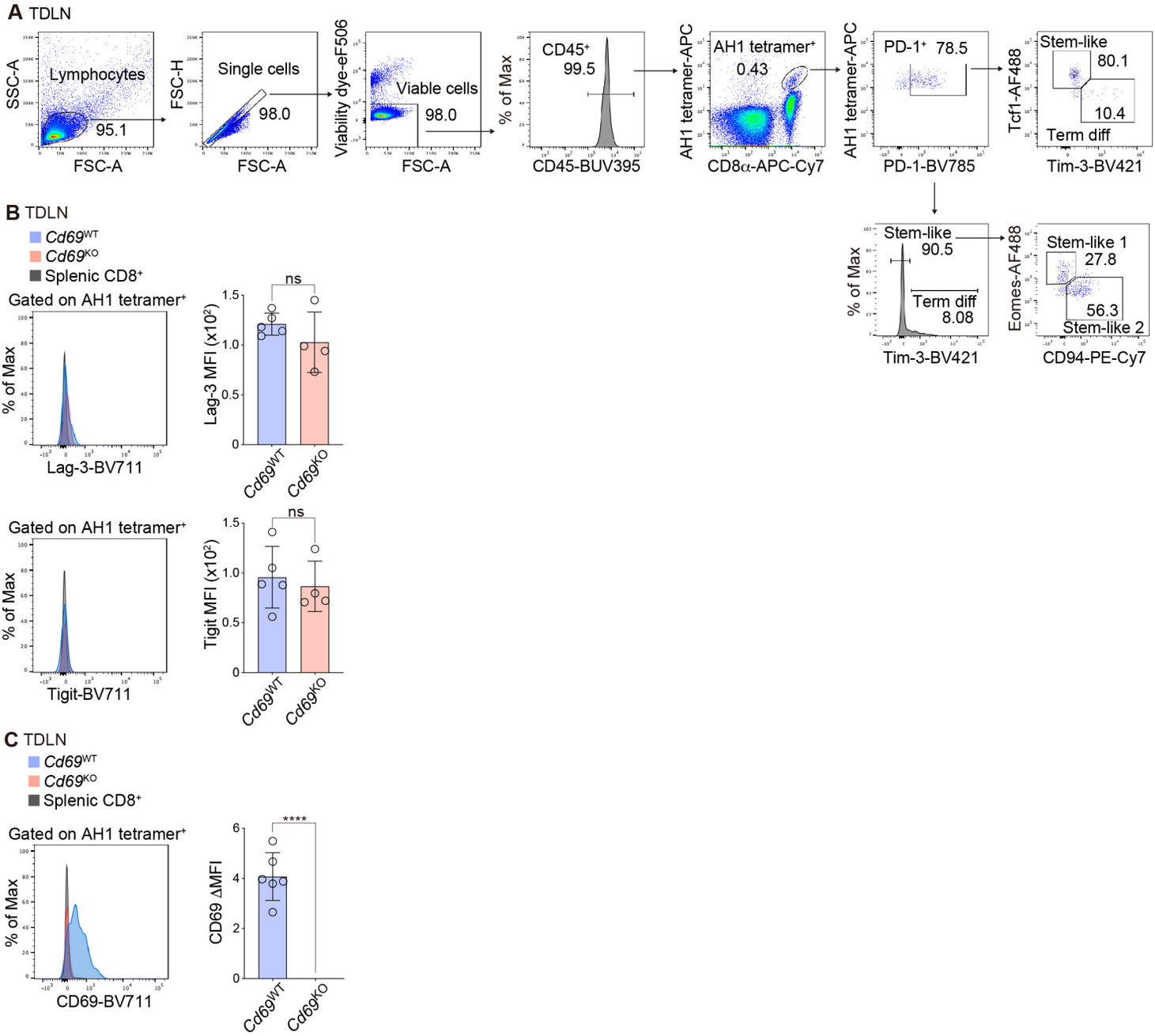
D TME



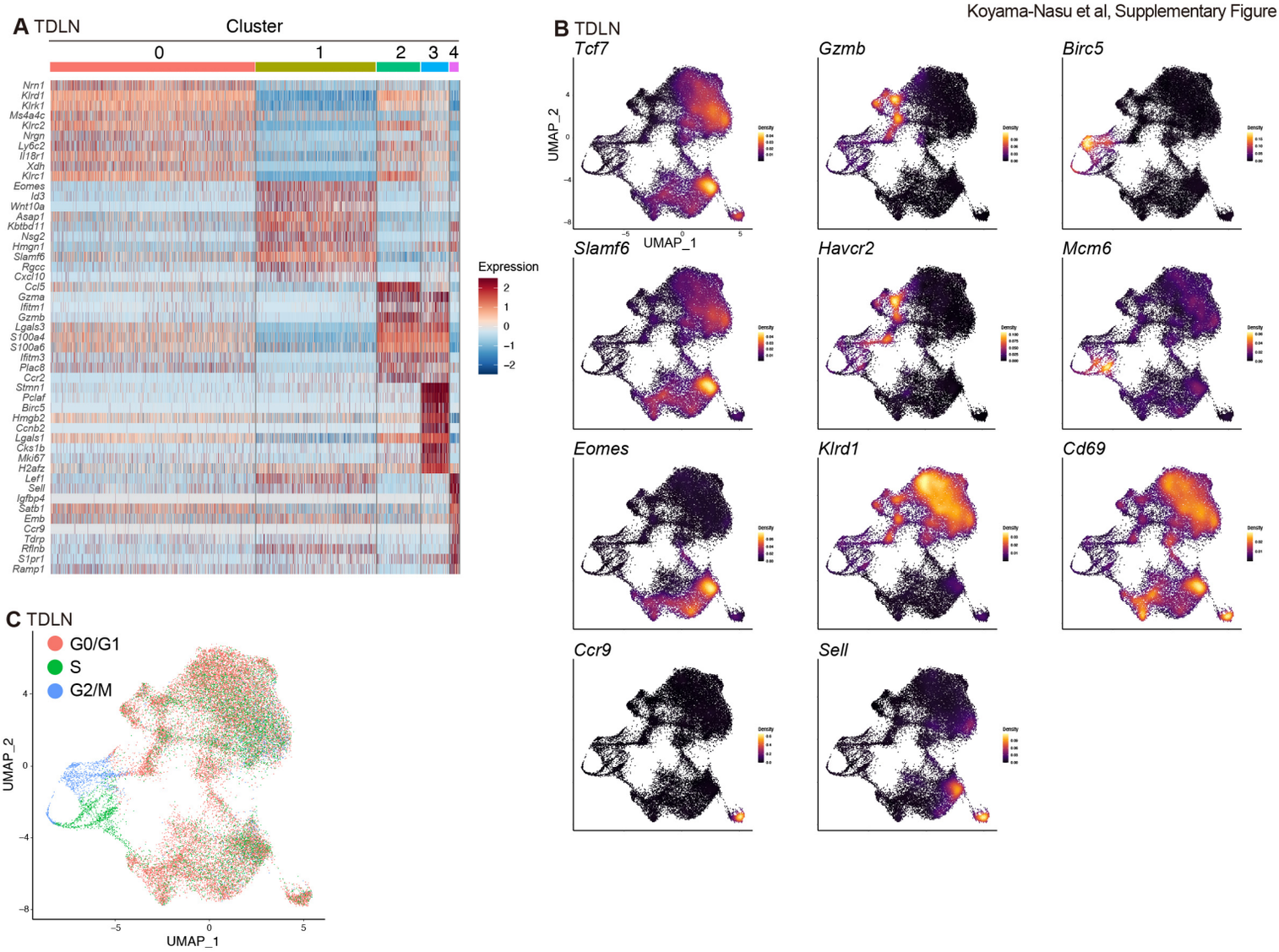
E TME



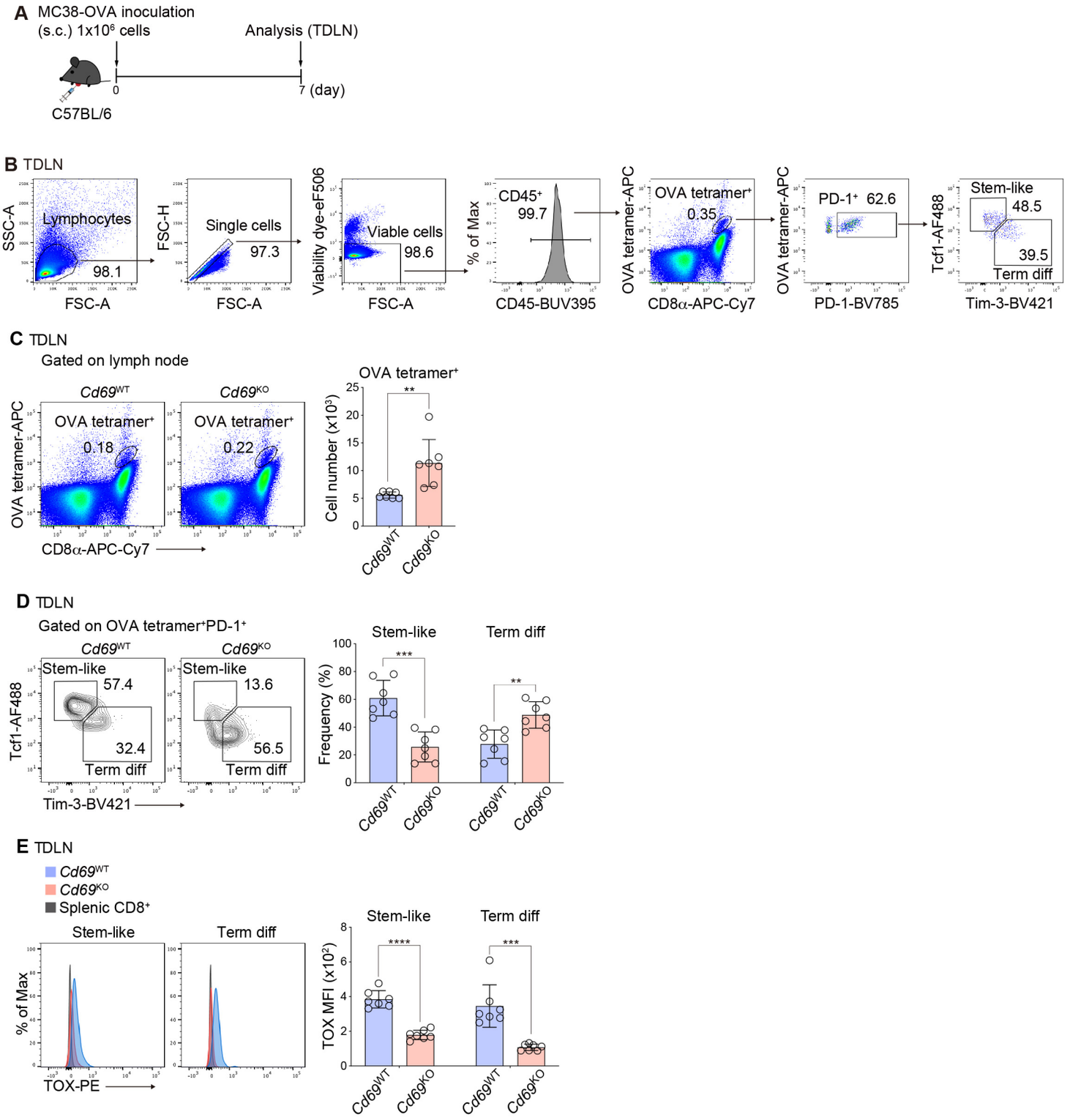
Supplementary Figure S4. CD69 controls the expression of the transcription factor TOX and TOX-mediated transcriptional program. **A,** A Venn diagram showing the overlap among genes encoding transcription factors downregulated in *Cd69*^{KO} stem-like CD8^{+} T cells and those downregulated in *Cd69*^{KO} terminally differentiated CD8^{+} T cells (left), showing the overlap among genes encoding transcription factors upregulated in *Cd69*^{KO} stem-like CD8^{+} T cells and those upregulated in *Cd69*^{KO} terminally differentiated CD8^{+} T cells (right). **B**, A Venn diagram showing the overlap among genes downregulated in *Cd69*^{KO} stem-like CD8^{+} T cells and those downregulated in *Cd69*^{KO} terminally differentiated CD8^{+} T cells (left), showing the overlap among genes upregulated in *Cd69*^{KO} stem-like CD8^{+} T cells and those upregulated in *Cd69*^{KO} terminally differentiated CD8^{+} T cells (right). **C**, CT26 cells were subcutaneously inoculated into *Cd69*^{WT} and *Cd69*^{KO} 7-*week-old* female BALB/c mice. Intratumoral AH1 tetramer^{+} CD8^{+} T cell subpopulations were sorted at 14 days after CT26 cell inoculation and subjected to Quantitative RT-PCR. n=3 per group. The graph represents relative fold change of *Tox* mRNA. **D**, A GSEA of the transcriptional signature from *Tox*^{WT} (left) or *Tox*^{KO} (right) Tim-3$^{–}$ (stem-like) cells in LCMV clone-13 infection in the ranked list of genes differentially expressed in AH1 tetramer^{+} stem-like CD8^{+} T cells in *Cd69*^{KO} mice versus *Cd69*^{WT} mice. **E**, A GSEA of the transcriptional signature from *Tox*^{WT} (left) or *Tox*^{KO} (right) Tim-3^{+} (terminally differentiated) cells in LCMV clone-13 infection in the ranked list of genes differentially expressed in AH1 tetramer^{+} terminally differentiated CD8^{+} T cells in *Cd69*^{KO} mice versus *Cd69*^{WT} mice. The gene expression profiles of *Tox*^{WT} and *Tox*^{KO} CD8^{+} T cell subpopulations in LCMV clone-13 infection were obtained from Alfei et al. (30). NES, normalized enrichment score. FDR, false discovery rate. An unpaired two-sided Student's *t*-test was used for the analysis of the quantitative RT-PCR data. * $P<0.05$, ** $P<0.01$.


A TDLN
SSC-A
Lymphocytes
95.1
FSC-A
FSC-H
Single cells
98.0
FSC-A
Viability dye-eF506
Viable cells
98.0
FSC-A
% of Max
CD45+
99.5
CD45-BUV395
AH1 tetramer-APC
AH1 tetramer+
0.43
CD8α-APC-Cy7
AH1 tetramer-APC
PD-1+ 78.5
PD-1-BV785
Tcf1-AF488
Stem-like
80.1
10.4
Term diff
Tim-3-BV421
B TDLN
Cd69WT
Cd69KO
Splenic CD8+
Gated on AH1 tetramer+
% of Max
Lag-3-BV711
Lag-3 MFI (x10²)
ns
Cd69WT
Cd69KO
Gated on AH1 tetramer+
% of Max
Tigit-BV711
Tigit MFI (x10²)
ns
Cd69WT
Cd69KO
% of Max
Stem-like
90.5
Term diff
8.08
Tim-3-BV421
Eomes-AF488
Stem-like 1
27.8
56.3
Stem-like 2
CD94-PE-Cy7
C TDLN
Cd69WT
Cd69KO
Splenic CD8+
Gated on AH1 tetramer+
% of Max
CD69-BV711
CD69 ΔMFI
****
Cd69WT
Cd69KO

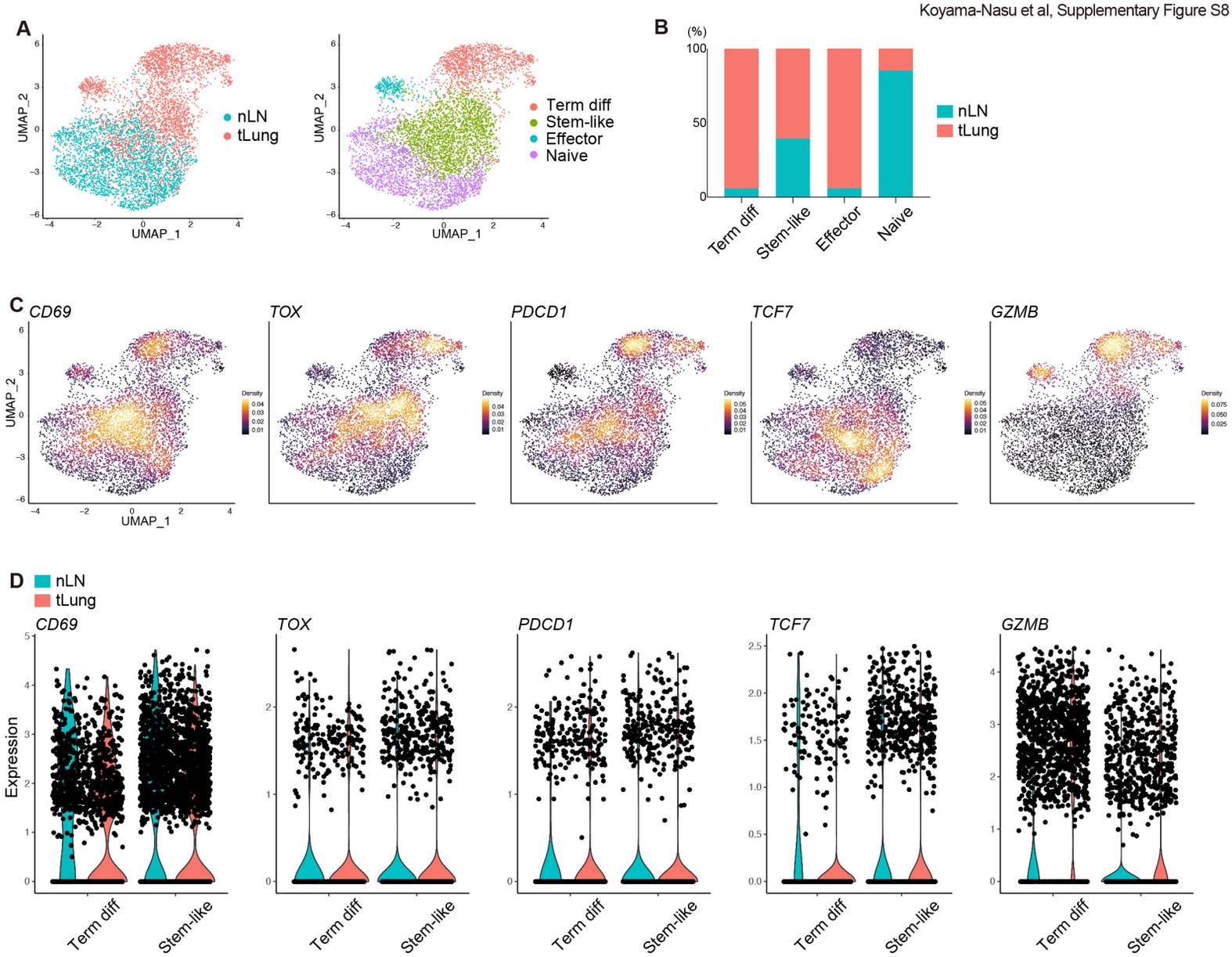
Supplementary Figure S5. Flow cytometry of tumor-specific $CD8^+$ T cells in TDLNs. **A**, Representative flow cytometry gating strategy for $CD8^+$ T cell subpopulations in TDLNs. CT26 cells were subcutaneously inoculated into 8-week-old female BALB/c mice. AH1 $tetramer^+$ $CD8^+$ T cells in TDLNs were analyzed at 14 days after inoculation. **B**, CT26 cells were subcutaneously inoculated into *Cd69*^{WT} and *Cd69*^{KO} 8-week-old female BALB/c mice. AH1 $tetramer^+$ $CD8^+$ T cells in TDLNs were analyzed at 14 days after inoculation. At least n=4 per group. MFI of the indicated proteins in $CD8^+$ T cells. A representative histogram (left) and summary of the experiments (right). Splenic $CD8^+$ cells are shown as a control. **C**, Delta MFI of CD69 (delta MFI was calculated by subtracting splenic $CD8^+$ MFI from AH1-specific $CD8^+$ T MFI). An unpaired two-sided Student's *t*-test was used for the analysis of the flow cytometry data. **** $P<0.0001$. ns: not significant. Representative data from two experiments with at least four mice are shown (**B-C**).


A TDLN
Cluster
0
1
2
3
4
Nrn1
Klrd1
Klrk1
Ms4a4c
Klrc2
Nrgn
Ly6c2
Il18r1
Xdh
Klrc1
Eomes
Id3
Wnt10a
Asap1
Kbtbd11
Nsg2
Hmgn1
Slamf6
Rgcc
Cxcl10
Ccl5
Gzma
Ifitm1
Gzmb
Lgals3
S100a4
S100a6
Ifitm3
Plac8
Ccr2
Stmn1
Pclaf
Birc5
Hmgb2
Ccnb2
Lgals1
Cks1b
Mki67
H2afz
Lef1
Sell
Igfbp4
Satb1
Emb
Ccr9
Tdrp
Rftnb
S1pr1
Ramp1
Expression
2
1
0
−1
−2
B TDLN
Tcf7
Gzmb
Birc5
Slamf6
Havcr2
Mcm6
Eomes
Klrd1
Cd69
Ccr9
Sell
UMAP_2
UMAP_1
C TDLN
G0/G1
S
G2/M
UMAP_2
UMAP_1
D CT26 inoculation
(s.c.) 1x10⁶ cells
Analysis (TDLN)
0
4
8
14 (day)
BALB/c
E TDLN 14 days
Gated on AH1 tetramer⁺PD-1⁺
Cd69WT
Cd69KO
Stem-like
88.5
5.88
Term diff
Stem-like
68.6
21.1
Term diff
Tcf1-AF488
Tim-3-BV421
Stem-like Term diff
Stem-like Term diff
Stem-like Term diff
Frequency (%)
100
50
0
ns
ns
**
*
n.d.
Cd69WT
Cd69KO
Cd69WT
Cd69KO
Cd69WT
Cd69KO
Cd69WT
Cd69KO
Cd69WT
Cd69KO
Cd69WT
Cd69KO
4 days
8 days
14 days
F TDLN 14 days
Cd69WT
Cd69KO
Splenic CD8⁺
Gated on AH1 tetramer⁺PD-1⁺
% of Max
TOX-PE
TOX MFI (x10²)
8
6
4
2
0
*
**
n.d.
Cd69WT
Cd69KO
Cd69WT
Cd69KO
Cd69WT
Cd69KO
4 days
8 days
14 days

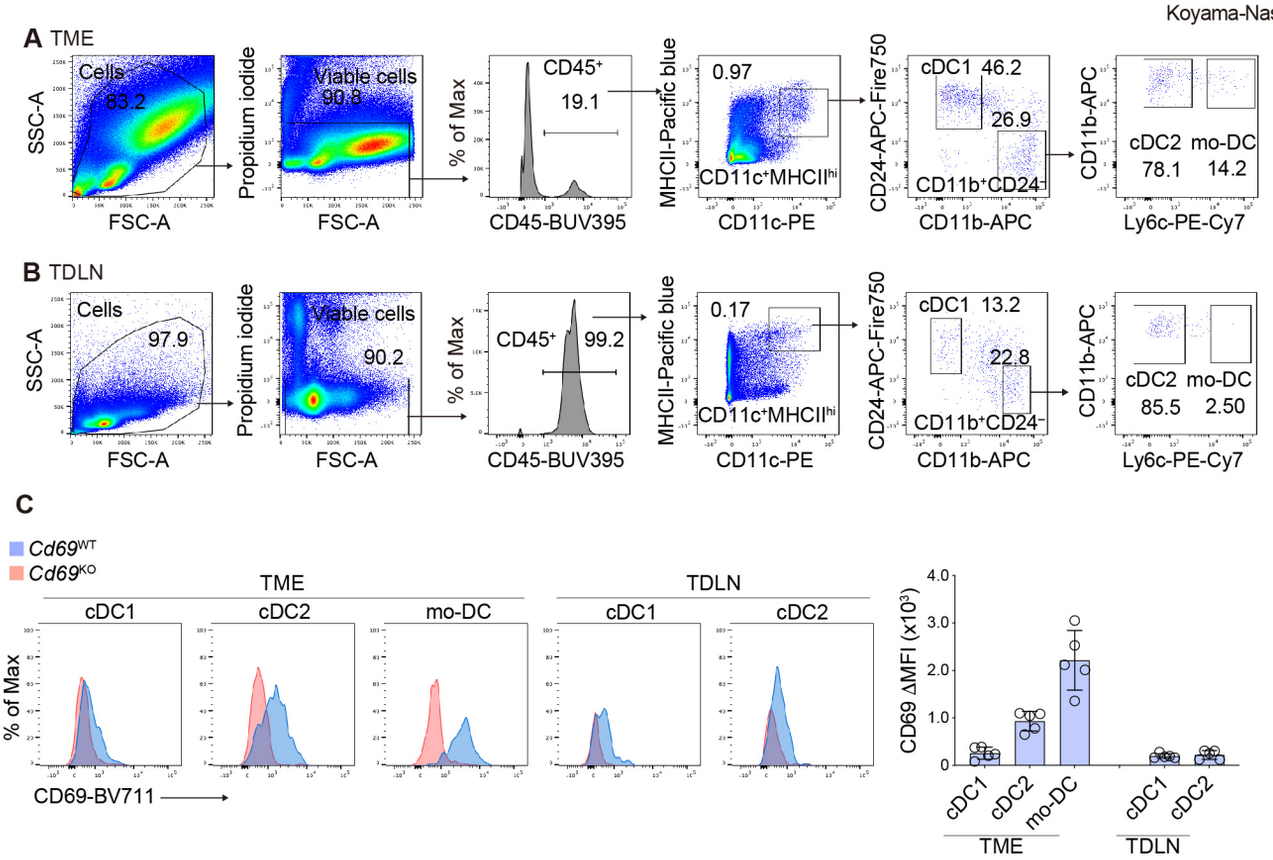
Supplementary Figure S6. Analysis of tumor-specific $CD8^+$ T cells in TDLNs. scRNA-seq. **A**, CT26 cells were subcutaneously inoculated into *Cd69*^{WT} and *Cd69*^{KO} 6-week-old female BALB/c mice. AH1 tetramer$^+$ $CD8^+$ T cells in TDLNs were isolated at 14 days after inoculation. Experiments were performed in duplicate. Pool of 10 mice in 1 group. A UMAP projection of *Cd69*^{WT} (n=7,014 and 5,807 cells) and *Cd69*^{KO} (n=6,285 and 10,956 cells) cells that were assigned to 5 clusters and color-coded based on the clusters, determined by Seurat. A heatmap of the top 10 genes expressed in each cluster as defined in Fig. 3B. The columns correspond to the cells, the rows correspond to the genes. Cells are grouped by clusters. The color scale is based on a z-score distribution from -2 (blue) to 2 (red). **B**, Single-cell transcript levels of the indicated genes illustrated in the UMAP plots. **C**, Cell-cycle phase (G0/G1, S, or G2/M) of each cell was determined by Seurat and is shown in the UMAP plots. **D**, A schematic outline of CT26 tumor model. **E-F**, CT26 cells were subcutaneously inoculated into 9-week-old female BALB/c mice. AH1 tetramer$^+$ $CD8^+$ T cells in TDLNs were analyzed at the indicated time. At least n=3 per group. The frequency of $CD8^+$ T cell subpopulations in AH1 tetramer$^+$PD-1^+ $CD8^+$ T cells (**E**). TOX MFI in AH1 tetramer$^+$PD-1^+ $CD8^+$ T cells (**F**). An unpaired two-sided Student's *t*-test was used for the analysis of the flow cytometry data. * $P<0.05$, ** $P<0.01$. ns: not significant. Representative data from two experiments with at least three mice are shown. n.d. means not detected.


A
MC38-OVA inoculation
(s.c.) 1x10⁶ cells
Analysis (TDLN)
0
7 (day)
C57BL/6
B TDLN
SSC-A
Lymphocytes
98.1
FSC-A
FSC-H
Single cells
97.3
FSC-A
Viability dye-eF506
Viable cells
98.6
FSC-A
% of Max
CD45⁺
99.7
CD45-BUV395
OVA tetramer-APC
OVA tetramer⁺
0.35
CD8α-APC-Cy7
OVA tetramer-APC
PD-1⁺ 62.6
PD-1-BV785
Tcf1-AF488
Stem-like
48.5
39.5
Term diff
Tim-3-BV421
C TDLN
Gated on lymph node
Cd69WT
Cd69KO
OVA tetramer-APC
OVA tetramer⁺
0.18
OVA tetramer⁺
0.22
CD8α-APC-Cy7
OVA tetramer⁺
Cell number (x10³)
**
Cd69WT
Cd69KO
D TDLN
Gated on OVA tetramer⁺PD-1⁺
Cd69WT
Cd69KO
Stem-like
57.4
32.4
Term diff
Stem-like
13.6
56.5
Term diff
Tcf1-AF488
Tim-3-BV421
Stem-like
Term diff
Frequency (%)
***
**
Cd69WT
Cd69KO
Cd69WT
Cd69KO
E TDLN
Cd69WT
Cd69KO
Splenic CD8⁺
Stem-like
Term diff
% of Max
TOX-PE
Stem-like
Term diff
TOX MFI (x10²)
****
***
Cd69WT
Cd69KO
Cd69WT
Cd69KO

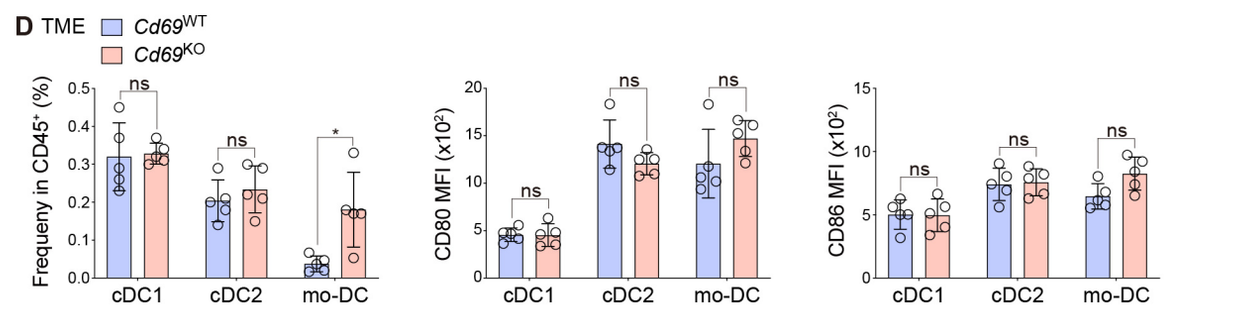
Supplementary Figure S7. Flow cytometry of OVA-specific $CD8^+$ T cells in TDLNs. **A**, A schematic outline of MC38-OVA tumor model. **B,** A representative flow cytometry gating strategy for $CD8^+$ T cell subpopulations in TDLNs. MC38-OVA cells were subcutaneously inoculated into 8-week-old C57BL/6 mice. OVA $tetramer^+$ $CD8^+$ T cells in TDLNs were analyzed at seven days after inoculation. **C-E**, MC38-OVA cells were subcutaneously inoculated into *Cd69*^{WT} and *Cd69*^{KO} 8-week-old C57BL/6 mice. OVA $tetramer^+$ $CD8^+$ T cells in TDLNs were analyzed at seven days after inoculation. n=7 per group. The frequency of OVA $tetramer^+$ $CD8^+$ T cells in $CD45^+$ cells. The number indicates the percentage of gated cells. Representative flow plots (left) and a summary of the experiments (right) (**C**). The frequency of $CD8^+$ T cell subpopulations in OVA $tetramer^+PD\text{-}1^+$ $CD8^+$ T cells (**D**). TOX MFI in $CD8^+$ T cell subpopulations (**E**). An unpaired two-sided Student's *t*-test was used for the analysis of the flow cytometry data. ** P<0.01, *** P<0.001, **** P<0.0001. Representative data from two experiments with at least five mice are shown (**C-E**).

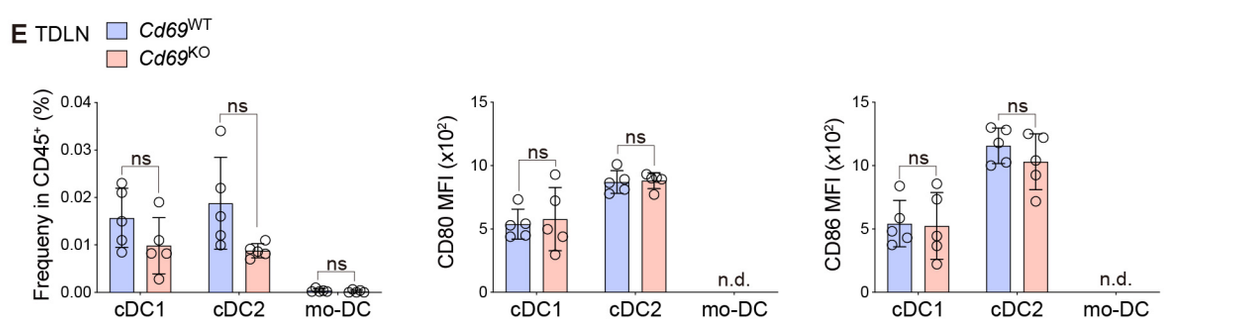

A
UMAP_2
UMAP_1
nLN
tLung
Term diff
Stem-like
Effector
Naive
B
(%)
100
50
0
nLN
tLung
Term diff
Stem-like
Effector
Naive
C
CD69
TOX
PDCD1
TCF7
GZMB
Density
D
nLN
tLung
Expression
Term diff
Stem-like

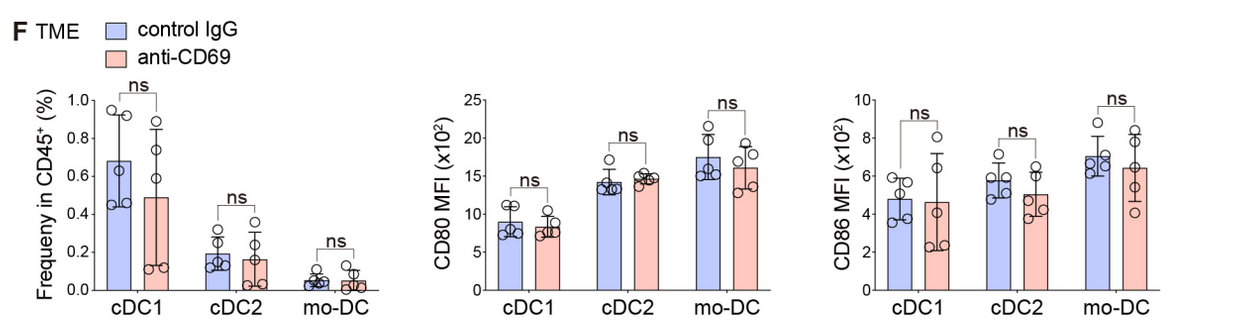
Supplementary Figure S8. The expression of *CD69* and *TOX* in $CD8^+$ T cells in human TDLNs. **A**, A UMAP projection of $CD8^+$ T cell (CD8 low T, Cytotoxic CD8+ T, Naive CD8+ T and Exhausted CD8+ T) clusters in treatment-naïve human lung adenocarcinoma patients from tumor-containing lung tissue (tLung) and non-metastatic lung-draining lymph nodes (nLN). **B**, The graph represents the percentages of cells from the indicated tissues in each cluster. **C**, The single-cell transcript levels of the indicated genes illustrated in the UMAP plots. **D**, Violin plots showing the expression of the indicated genes in cells from stem-like and term diff clusters in both nLN and tLung as defined in **A**. Each dot represents a single cell. The scRNA-seq dataset was obtained from Kim et al. (35).

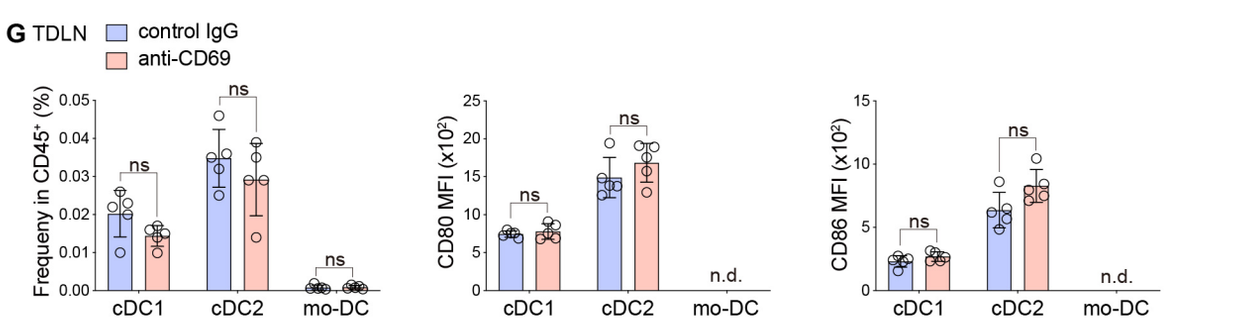

A TME
Cells
83.2
SSC-A
FSC-A
Propidium iodide
Viable cells
90.8
FSC-A
% of Max
CD45+
19.1
CD45-BUV395
0.97
MHCII-Pacific blue
CD11c+MHCIIhi
CD11c-PE
CD24-APC-Fire750
cDC1 46.2
26.9
CD11b+CD24-
CD11b-APC
CD11b-APC
cDC2 78.1
mo-DC 14.2
Ly6c-PE-Cy7
B TDLN
Cells
97.9
Viable cells
90.2
CD45+ 99.2
0.17
cDC1 13.2
22.8
cDC2 85.5
mo-DC 2.50

C
Cd69WT
Cd69KO
TME
TDLN
cDC1
cDC2
mo-DC
% of Max
CD69-BV711
CD69 ΔMFI (x10³)
4.0
3.0
2.0
1.0
0

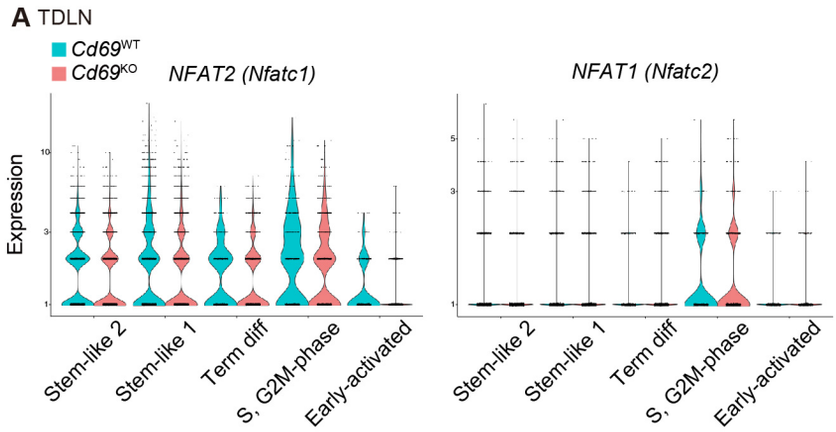

D TME
Cd69WT
Cd69KO
Frequeny in CD45+ (%)
CD80 MFI (x10²)
CD86 MFI (x10²)
ns
*
cDC1
cDC2
mo-DC

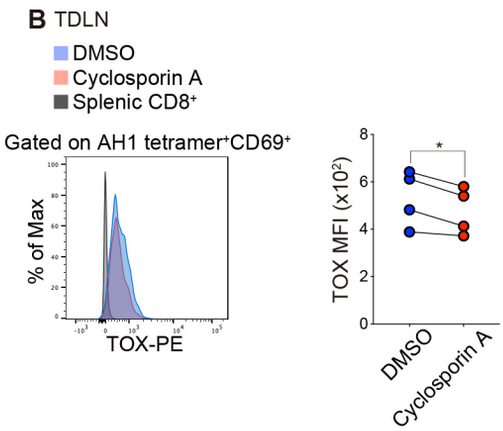

E TDLN
Cd69WT
Cd69KO
Frequeny in CD45+ (%)
CD80 MFI (x10²)
CD86 MFI (x10²)
ns
n.d.
cDC1
cDC2
mo-DC

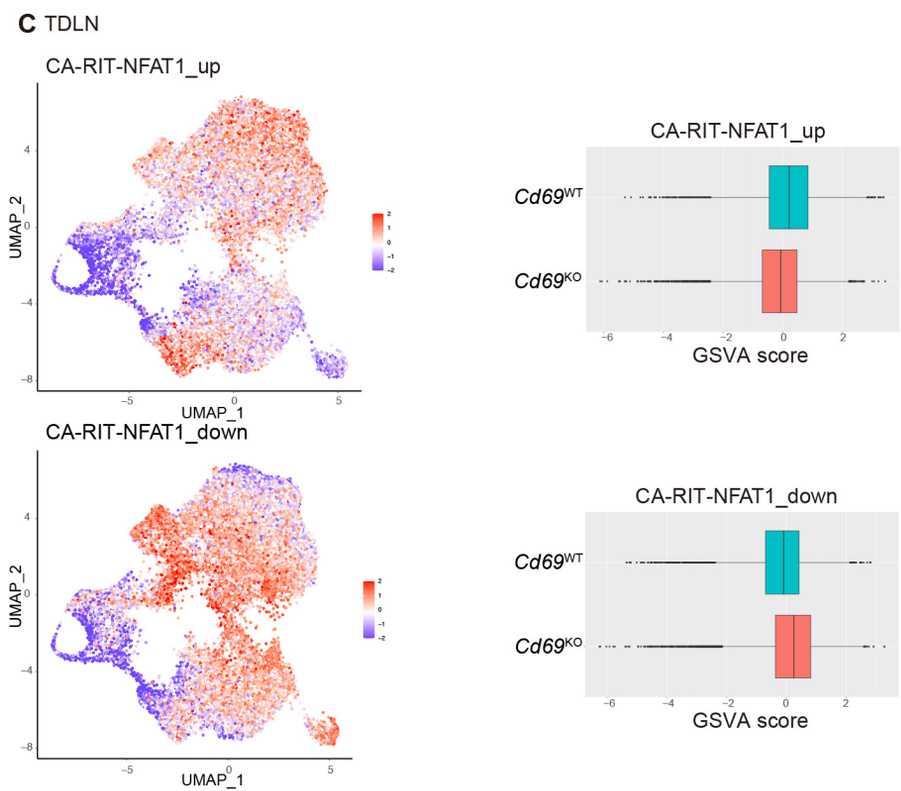

F TME
control IgG
anti-CD69
Frequeny in CD45+ (%)
CD80 MFI (x10²)
CD86 MFI (x10²)
ns
cDC1
cDC2
mo-DC


G TDLN
control IgG
anti-CD69
Frequeny in CD45+ (%)
CD80 MFI (x10²)
CD86 MFI (x10²)
ns
n.d.
cDC1
cDC2
mo-DC

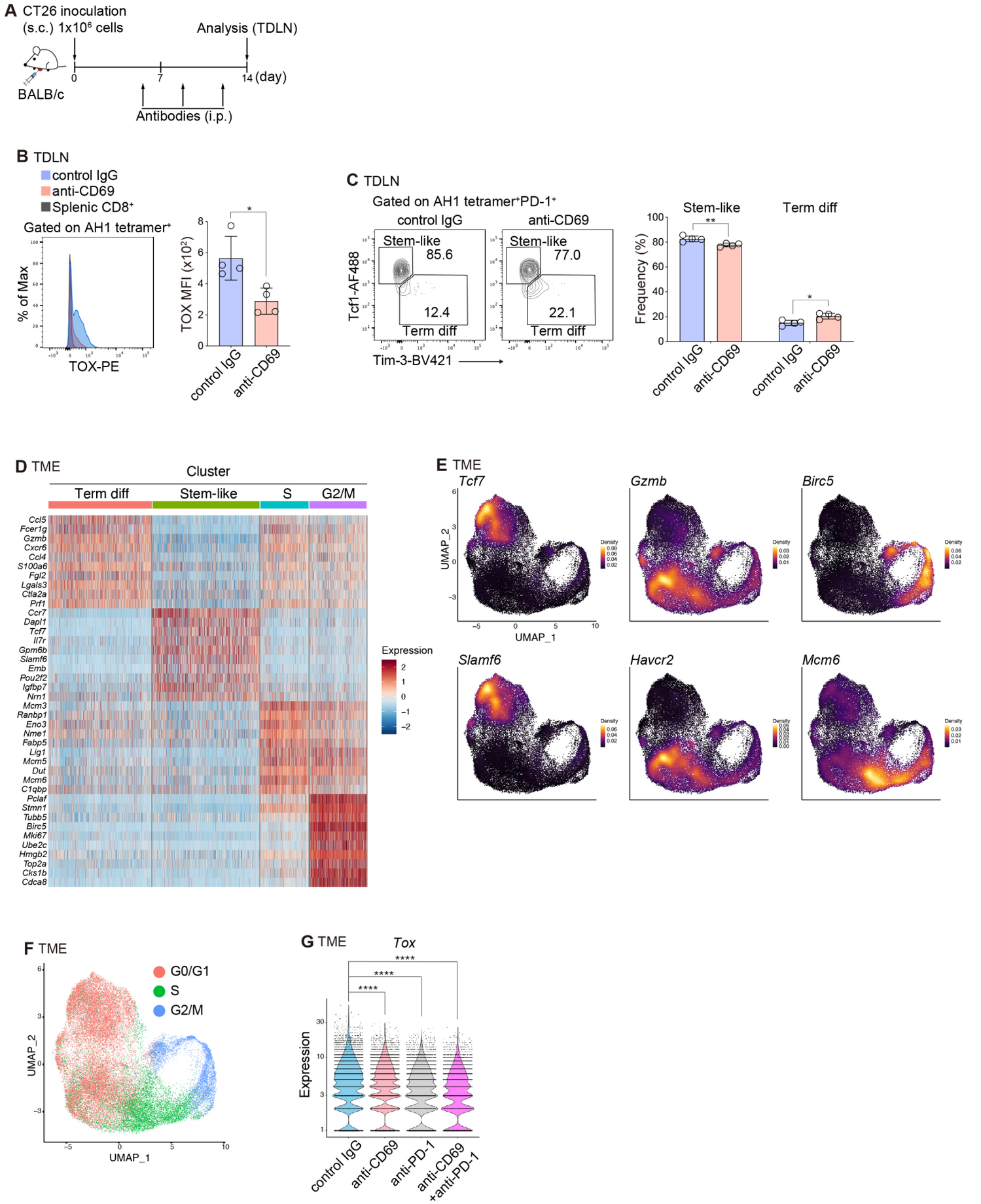
Supplementary Figure S9. Analysis of major dendritic cell populations in the TME and TDLNs. A representative flow cytometry gating strategy for major dendritic cell (DC) populations in the TME (**A**) and TDLNs (**B**). **C**, CT26 cells were subcutaneously inoculated into 12-week-old BALB/c mice. n=5 per group. Delta MFI of CD69 (delta MFI was calculated by subtracting *Cd69*^{KO} MFI from *Cd69*^{WT} MFI) in cDC1, cDC2 and mo-DCs in the TME and cDC1 and cDC2 in TDLNs. **D-E**, CT26 cells were subcutaneously inoculated into *Cd69*^{WT} and *Cd69*^{KO} 12-week-old female BALB/c mice. n=5 per group. DCs in the TME (**D**) and TDLNs (**E**) were analyzed by flow cytometry. The frequency of DCs in CD45^{+} cells and MFI of the indicated protein were shown. **F-G**, CT26 cells were subcutaneously inoculated into 6-week-old BALB/c mice. n=5 per group. Anti-CD69 Ab or control IgG were intraperitoneally administered to mice twice a week, 3 times. DCs in the TME (**F**) and TDLNs (**G**) were analyzed by flow cytometry. The frequency of DCs in CD45^{+} cells and MFI of the indicated protein were shown. n.d. means not detected. An unpaired two-sided Student's *t*-test was used for the analysis of the flow cytometry data. * $P<0.05$. not significant. Representative data from two experiments with at least five mice are shown (**C-G**).


A TDLN
Cd69WT
Cd69KO
NFAT2 (Nfatc1)
NFAT1 (Nfatc2)
Expression
Stem-like 2
Stem-like 1
Term diff
S, G2M-phase
Early-activated


B TDLN
DMSO
Cyclosporin A
Splenic CD8+
Gated on AH1 tetramer+CD69+
% of Max
TOX-PE
TOX MFI (x10²)
*
DMSO
Cyclosporin A


C TDLN
CA-RIT-NFAT1_up
UMAP_2
UMAP_1
CA-RIT-NFAT1_up
Cd69WT
Cd69KO
GSVA score
CA-RIT-NFAT1_down
UMAP_2
UMAP_1
CA-RIT-NFAT1_down
Cd69WT
Cd69KO
GSVA score

Supplementary Figure S10. CD69 controls NFAT signaling in TDLNs. **A**, Violin plots showing the expression of the indicated genes in cells from each cluster as defined in Fig. 3B. The violin represents the probability density at each value. Each dot represents a single cell. **B**, CT26 cells were subcutaneously inoculated into *Cd69*^{WT} and *Cd69*^{KO} 6-week-old female BALB/c mice. n=4 per group. TDLN cells were cultured *in vitro* in the presence or absence of Cyclosporin A for 4 hours. MFI of TOX in AH1 tetramer^{+}CD69^{+} CD8^{+} T cells. **C**, Single-sample gene set variation analysis (ssGSVA) scores of CA-RIT-NFAT1_up (upper) and CA-RIT-NFAT1_down (lower) are projected on UMAPs and box plots. The gene expression profiles of CA-RIT-NFAT1- and mock-transduced CD8^{+} T cells were obtained from Martinez et al.(26). An unpaired two-sided Student's *t*-test was used for the analysis of the flow cytometry data. * P<0.05. Representative data from two experiments with at least four mice are shown (**B**).


A
CT26 inoculation
(s.c.) 1x10⁶ cells
Analysis (TDLN)
BALB/c
0
7
14 (day)
Antibodies (i.p.)
B TDLN
control IgG
anti-CD69
Splenic CD8⁺
Gated on AH1 tetramer⁺
% of Max
TOX-PE
TOX MFI (x10²)
control IgG
anti-CD69
C TDLN
Gated on AH1 tetramer⁺PD-1⁺
control IgG
anti-CD69
Stem-like
85.6
12.4
Term diff
77.0
22.1
Tcf1-AF488
Tim-3-BV421
Frequency (%)
D TME
Cluster
Term diff
Stem-like
S
G2/M
Ccl5
Fcer1g
Gzmb
Cxcr6
Ccl4
S100a6
Fgl2
Lgals3
Ctla2a
Prf1
Ccr7
Dapl1
Tcf7
Il7r
Gpm6b
Slamf6
Emb
Pou2f2
Igfbp7
Nrn1
Mcm3
Ranbp1
Eno3
Nme1
Fabp5
Lig1
Mcm5
Dut
Mcm6
C1qbp
Pclaf
Stmn1
Tubb5
Birc5
Mki67
Ube2c
Hmgb2
Top2a
Cks1b
Cdca8
Expression
E TME
Tcf7
Gzmb
Birc5
Slamf6
Havcr2
Mcm6
UMAP_1
UMAP_2
Density
F TME
G0/G1
S
G2/M
UMAP_1
UMAP_2
G TME
Tox
Expression
control IgG
anti-CD69
anti-PD-1
anti-CD69 +anti-PD-1
H TME
SSC-A
Lymphocytes
38.7
FSC-A
FSC-H
Single cells
97.2
FSC-A
Viability dye-eF506
Viable cells
89.4
FSC-A
% of Max
CD45⁺
91.3
CD45.2-PE
OVA tetramer-APC
OVA tetramer⁺
1.24
CD8α-APC-Cy7
OVA tetramer-APC
PD-1⁺ 90.8
PD-1-BV421
Tcf1-AF488
Stem-like
47.3
40.9
Term diff
Tim-3-PE-Cy7

Supplementary Figure S11. Analysis of tumor-specific $CD8^+$ T cells from mice treated with therapeutic antibodies. **A**, A schematic outline of the administration of antibodies during CT26 tumor development. CT26 cells were subcutaneously inoculated into 6-week-old female BALB/c mice. Antibodies were intraperitoneally administered to mice twice a week, 3 time. AH1 tetramer$^+$ $CD8^+$ T cells in TDLNs were analyzed at 14 days after CT26 cell inoculation. n=4 per group. TOX MFI (**B**). The frequency of $CD8^+$ T cell subpopulations (**C**). **D-F**, scRNA-seq. CT26 cells were subcutaneously inoculated into 7-week-old female BALB/c mice. Abs were intraperitoneally administered to mice twice a week, 3 times. Intratumoral AH1 tetramer$^+$ $CD8^+$ T cells were isolated at 14 days after inoculation. Experiments were performed in duplicate. Pool of four mice in 1 group. A UMAP projection of control IgG (n=6,430 and 6,489 cells), anti-CD69 (n=5,933 and 6,213 cells), anti-PD-1 (n=6,706 and 4,950 cells) and anti-CD69 and anti-PD-1 (n=7,094 and 3,674 cells) treated cells that were assigned to 4 clusters and color-coded based on the clusters, determined by Seurat. **D**, A heatmap of the top 10 genes expressed in each cluster as defined in Fig. 5B. The columns correspond to the cells, the rows correspond to the genes. Cells are grouped by clusters. The color scale is based on a z-score distribution from -2 (blue) to 2 (red). **E**, Single-cell transcript levels of the indicated genes illustrated in the UMAP plots. **F**, The cell-cycle phase (G0/G1, S, or G2/M) of each cell was determined by Seurat and is shown in the UMAP plots. **G**, Violin plots showing the expression of the *Tox* gene in cells treated with the indicated antibodies. The violin represents the probability density at each value. Each dot represents a single cell. Statistical test is performed using one-way ANOVA. **H**, A representative flow cytometry gating strategy for intratumoral $CD8^+$ T cell subpopulations. B16-OVA cells were subcutaneously inoculated into 7-week-old C57BL/6 mice. Intratumoral OVA tetramer$^+$ $CD8^+$ T cells were analyzed at 10 days after inoculation. An unpaired two-sided Student's *t*-test was used for the analysis of the flow cytometry data. * $P<0.05$, ** $P<0.01$. Representative data from two experiments with at least four mice are shown (**B-C**).