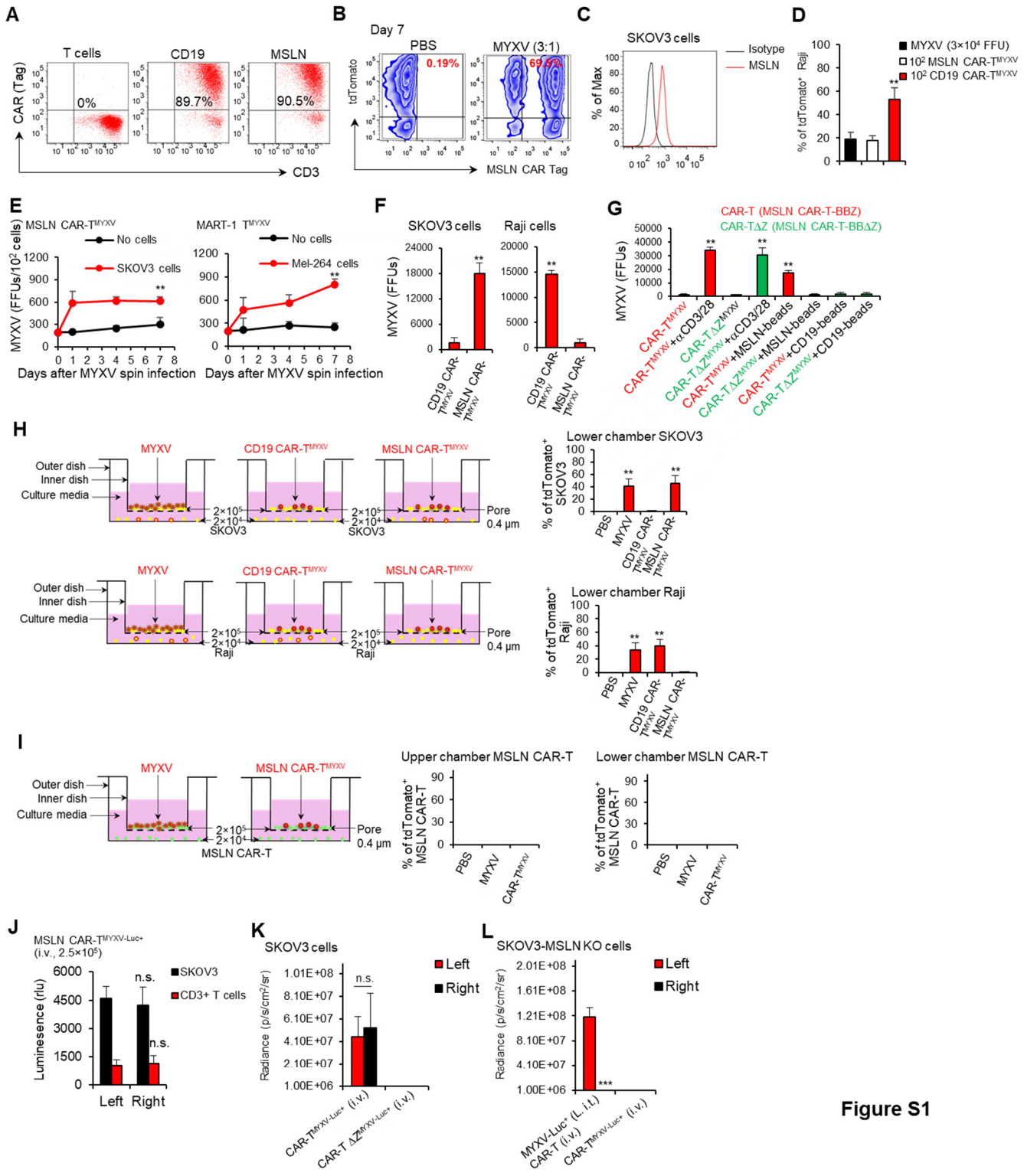
A
T cells
CD19
MSLN
CAR (Tag)
CD3
0%
89.7%
90.5%
B
Day 7
PBS
MYXV (3:1)
tdTomato
0.19%
MSLN CAR Tag
C
SKOV3 cells
Isotype
MSLN
% of Max
D
MYXV (3×10⁴ FFU)
10² MSLN CAR-T^MYXV
10² CD19 CAR-T^MYXV
% of tdTomato⁺ Raji
E
MSLN CAR-T^MYXV
No cells
SKOV3 cells
MART-1 T^MYXV
Mel-264 cells
MYXV (FFUs/10² cells)
Days after MYXV spin infection
F
SKOV3 cells
Raji cells
MYXV (FFUs)
CD19 CAR-T^MYXV
MSLN CAR-T^MYXV
G
CAR-T (MSLN CAR-T-BBZ)
CAR-TΔZ (MSLN CAR-T-BBΔZ)
MYXV (FFUs)
CAR-T^MYXV
CAR-T^MYXV+αCD3/28
CAR-TΔZ^MYXV
CAR-TΔZ^MYXV+αCD3/28
CAR-T^MYXV+MSLN-beads
CAR-TΔZ^MYXV+MSLN-beads
CAR-T^MYXV+CD19-beads
CAR-TΔZ^MYXV+CD19-beads
H
MYXV
CD19 CAR-T^MYXV
MSLN CAR-T^MYXV
Outer dish
Inner dish
Culture media
2×10⁵
2×10⁴
SKOV3
Raji
Pore
0.4 μm
Lower chamber SKOV3
Lower chamber Raji
% of tdTomato⁺ SKOV3
% of tdTomato⁺ Raji
PBS
MYXV
CD19 CAR-T^MYXV
MSLN CAR-T^MYXV
I
MSLN CAR-T
Upper chamber MSLN CAR-T
Lower chamber MSLN CAR-T
% of tdTomato⁺ MSLN CAR-T
CAR-T^MYXV
J
MSLN CAR-T^MYXV-Luc+
(i.v., 2.5×10⁵)
SKOV3
CD3+ T cells
Luminescence (rlu)
n.s.
Left
Right
K
SKOV3 cells
Radiance (p/s/cm²/sr)
CAR-T^MYXV-Luc+ (i.v.)
CAR-T ΔZ^MYXV-Luc+ (i.v.)
L
SKOV3-MSLN KO cells
MYXV-Luc+ (i.v.)
CAR-T (i.v.)
CAR-T^MYXV-Luc+ (i.v.)

Figure S1

Figure S1. Characteristics of MYXV-loaded T cells. Related to Figure 1.

(**A**) γ-retrovirus encoding human MSLN BBZ CAR or CD19 BBZ CAR was transduced into the activated human T cells one day after activation. After expansion for 7 days, % of CD19 CAR$^+$ and MSLN CAR$^+$ T cells were detected by FACS. Representative results are shown.

(**B**) MYXV-tdTomato was added to CAR-T cells at MOI of 3:1 (MYXV:MSLN CAR-T) in the presence of 10 μg/ml protamine, and the culture was centrifuged at 1,800 *rpm* for 2 hrs for loading MYXV to CAR-T cells. The MYXV infection rate was detected by FACS 7 days after adding MYXV.

(**C**) The surface expression level of MSLN on SKOV3 cells was detected by FACS.

(**D**) MYXV-tdTomato (3×10^4 FFUs), CD19 CAR-T$^{MYXV\text{-}tdTomato}$, or MSLN CAR-T$^{MYXV\text{-}tdTomato}$ cells (1×10^2) were added to Raji cells (1×10^4). % of tdTomato$^+$ Raji cells are shown (24 hrs; n=3-5/group). $^{**}P<0.01$, CD19 CAR-T^{MYXV} compared with any other groups, one-way ANOVA with Tukey test.

(**E**-**G**) MYXV-tdTomato titers (or replication) were determined using red foci formation on BSC40 cells. Titers for each sample were performed in triplicate. (**E**) MSLN CAR-T$^{MYXV\text{-}tdTomato}$ or MART-1 T$^{MYXV\text{-}tdTomato}$ cells were cocultured with/without SKOV3 or Mel-264 cells, respectively. MSLN CAR-T$^{MYXV\text{-}tdTomato}$ or MART-1 T$^{MYXV\text{-}tdTomato}$ cells were isolated and lysed to determine MYXV replicates in MSLN CAR-T$^{MYXV\text{-}tdTomato}$ and TRP-1 T$^{MYXV\text{-}tdTomato}$ cells at indicated time points. $^{**}P<0.01$, one-way ANOVA with Tukey test. (**F**) MSLN CAR-T$^{MYXV\text{-}tdTomato}$ or CD19 CAR-T$^{MYXV\text{-}tdTomato}$ were cocultured with SKOV3 or Raji cells. Cell culture supernatant was collected after 72 hrs to quantify MYXV released from CAR-T$^{MYXV\text{-}tdTomato}$ cells. $^{**}P<0.01$, Student's *t*-test. (**G**) CAR-T^{MYXV} (MSLN CAR-T^{MYXV}) and CAR-TΔZ^{MYXV} (MSLN CAR-T-BBΔZ^{MYXV}) were reactivated with MSLN beads or anti-CD3/28 beads, and CD19 beads were used as a negative control. Cell culture supernatant was collected 72 hrs after reactivation to determine MYXV released from CAR-T^{MYXV} or CAR-TΔZ^{MYXV} cells. $^{**}P<0.01$, CAR-T^{MYXV}+αCD3/28 compared with CAR-T^{MYXV}, CAR-TΔZ ^{MYXV}+αCD3/28 compared with CAR-T^{MYXV}, CAR-T^{MYXV}+MSLN-beads compared with CAR-T^{MYXV}, one-way ANOVA with Tukey test.

(**H**) SKOV3 or Raji cells were seeded into the upper (2×10^5) and lower (2×10^4) Transwell chambers. MYXV-tdTomato (6×10^5 FFUs), CD19 CAR-T$^{MYXV\text{-}tdTomato}$, or MSLN CAR-T$^{MYXV\text{-}tdTomato}$ (2×10^3) cells were only added to upper chambers. % of tdTomato$^+$ SKOV3 or tdTomato$^+$ Raji in the lower chamber was detected after 48 hrs. $^{**}P<0.01$, MYXV compared with PBS (SKOV3), MSLN CAR-T^{MYXV} compared with PBS (SKOV3); MYXV compared with PBS (Raji), CD19 CAR-T^{MYXV} compared with PBS (Raji); One-way ANOVA with Tukey test. Representative results from one of two repeated experiments are shown.

(**I**) GFP$^+$ MSLN CAR-T cells were seeded into the upper (2×10^5) and lower (2×10^4) Transwell chambers. MYXV-tdTomato (6×10^4 FFUs), or GFP$^-$/tdTomato$^+$ MSLN CAR-T^{MYXV} cells (2×10^4) were only added to upper chambers. % of tdTomato$^+$GFP$^+$ MSLN CAR-T in the upper and lower chambers were detected after 48 hrs. Representative results from one of two repeated experiments are shown.

(**J**-**K**) NSG mice bearing 35-day established SKOV3 tumors. (**J**) MSLN CAR-T$^{MYXV\text{-}Luc+}$ cells were i.v. injected when tumors reached ~7×6 mm on day 35. Mice were euthanized on day 42. CD3$^+$ T cells were isolated from both flanks of tumors. Bioluminescence of SKOV3 cells (T cell-depleted) and CD3$^+$ T cells were detected. Pooled results represent 2 independent experiments. n.s. (not significant), Left compared with Right, two-way ANOVA with posthoc Holm-Sidak test. (**K**) MSLN CAR-T$^{MYXV\text{-}Luc+}$ or MSLN CAR-TΔZ$^{MYXV\text{-}Luc+}$ cells were i.v. injected on day 35. Bioluminescence of mice was measured on day 42. Summarized data are shown (n=3/group). n.s., Left compared with Right, one-way ANOVA with Tukey test.

(**L**) NSG mice bearing 35-day established MSLN-KO SKOV3 cells, indicated treatment schema was similar to Figure 1F. Bioluminescence of mice was measured on day 42. $^{***}P<0.001$, Left compared with Right, one-way ANOVA with Tukey test.

(**D**-**L**) Data are mean ± SD.

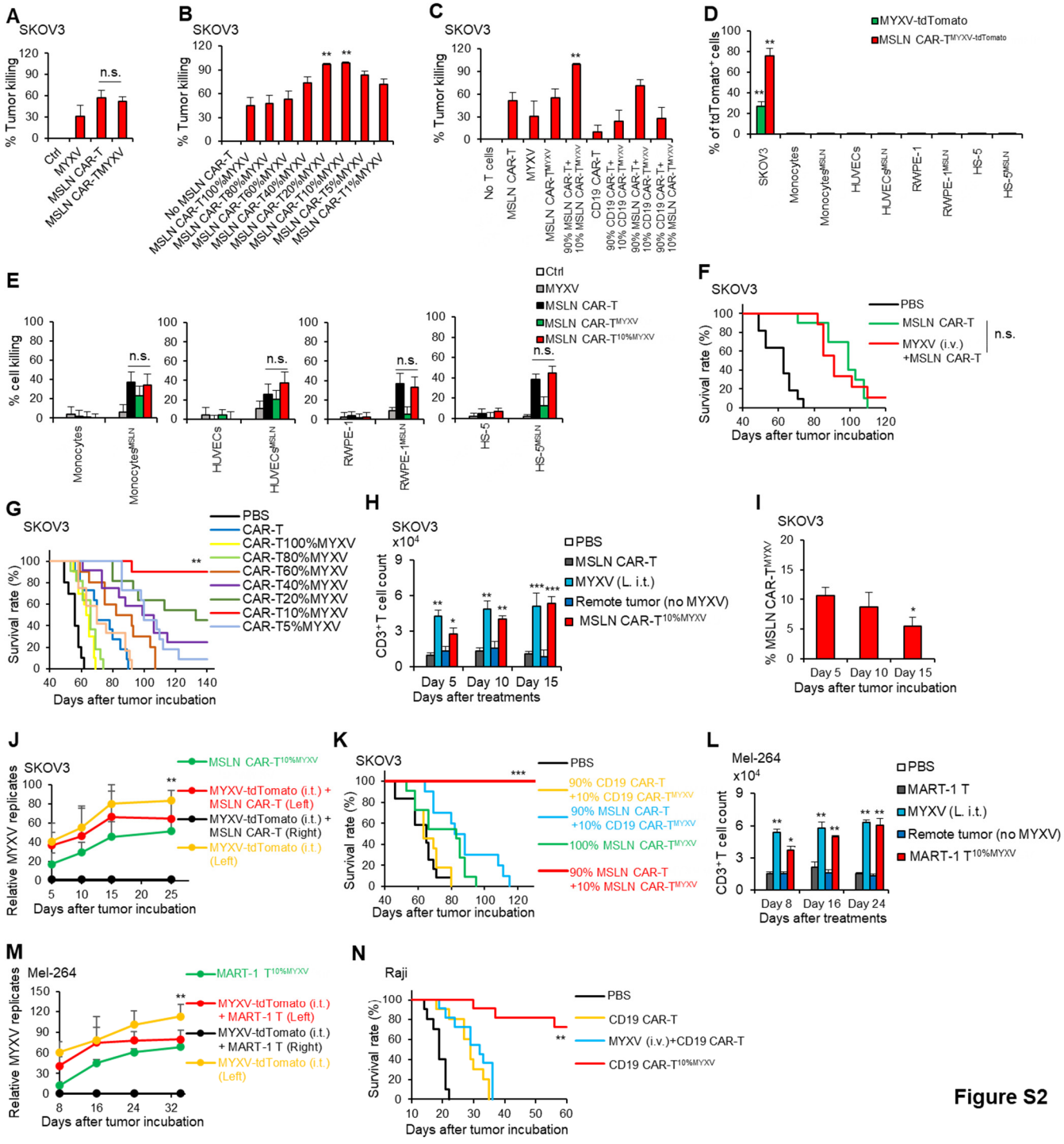
A
SKOV3
% Tumor killing
n.s.
Ctrl
MYXV
MSLN CAR-T
MSLN CAR-TMYXV
B
SKOV3
% Tumor killing
No MSLN CAR-T
MSLN CAR-T100%MYXV
MSLN CAR-T80%MYXV
MSLN CAR-T60%MYXV
MSLN CAR-T40%MYXV
MSLN CAR-T20%MYXV
MSLN CAR-T10%MYXV
MSLN CAR-T5%MYXV
MSLN CAR-T1%MYXV
C
SKOV3
% Tumor killing
No T cells
MSLN CAR-T
MYXV
MSLN CAR-TMYXV
90% MSLN CAR-T+ 10% MSLN CAR-TMYXV
CD19 CAR-T
90% CD19 CAR-T+ 10% CD19 CAR-TMYXV
90% MSLN CAR-T+ 10% CD19 CAR-TMYXV
90% CD19 CAR-T+ 10% MSLN CAR-TMYXV
D
MYXV-tdTomato
MSLN CAR-TMYXV-tdTomato
% of tdTomato+ cells
SKOV3
Monocytes
MonocytesMSLN
HUVECs
HUVECsMSLN
RWPE-1
RWPE-1MSLN
HS-5
HS-5MSLN
E
Ctrl
MYXV
MSLN CAR-T
MSLN CAR-TMYXV
MSLN CAR-T10%MYXV
% cell killing
n.s.
Monocytes
MonocytesMSLN
HUVECs
HUVECsMSLN
RWPE-1
RWPE-1MSLN
HS-5
HS-5MSLN
F
SKOV3
PBS
MSLN CAR-T
MYXV (i.v.) +MSLN CAR-T
n.s.
Survival rate (%)
Days after tumor incubation
G
SKOV3
PBS
CAR-T
CAR-T100%MYXV
CAR-T80%MYXV
CAR-T60%MYXV
CAR-T40%MYXV
CAR-T20%MYXV
CAR-T10%MYXV
CAR-T5%MYXV
Survival rate (%)
Days after tumor incubation
H
SKOV3
x10^4
CD3+ T cell count
PBS
MSLN CAR-T
MYXV (L. i.t.)
Remote tumor (no MYXV)
MSLN CAR-T10%MYXV
Day 5
Day 10
Day 15
Days after treatments
I
SKOV3
% MSLN CAR-TMYXV
Day 5
Day 10
Day 15
Days after tumor incubation
J
SKOV3
MSLN CAR-T10%MYXV
MYXV-tdTomato (i.t.) + MSLN CAR-T (Left)
MYXV-tdTomato (i.t.) + MSLN CAR-T (Right)
MYXV-tdTomato (i.t.) (Left)
Relative MYXV replicates
Days after tumor incubation
K
SKOV3
PBS
90% CD19 CAR-T +10% CD19 CAR-TMYXV
90% MSLN CAR-T +10% CD19 CAR-TMYXV
100% MSLN CAR-TMYXV
90% MSLN CAR-T +10% MSLN CAR-TMYXV
Survival rate (%)
Days after tumor incubation
L
Mel-264
x10^4
CD3+ T cell count
PBS
MART-1 T
MYXV (L. i.t.)
Remote tumor (no MYXV)
MART-1 T10%MYXV
Day 8
Day 16
Day 24
Days after treatments
M
Mel-264
MART-1 T10%MYXV
MYXV-tdTomato (i.t.) + MART-1 T (Left)
MYXV-tdTomato (i.t.) + MART-1 T (Right)
MYXV-tdTomato (i.t.) (Left)
Relative MYXV replicates
Days after tumor incubation
N
Raji
PBS
CD19 CAR-T
MYXV (i.v.)+CD19 CAR-T
CD19 CAR-T10%MYXV
Survival rate (%)
Days after tumor incubation

Figure S2

Figure S2. The optimal antitumor function requires CAR-T^{MYXV} tumor specificity. Related to Figure 2.

(**A**) SKOV3 cells (1×10^4) were treated with MYXV (3×10^4 FFUs), MSLN CAR-T, or MSLN CAR-T^{MYXV} (1×10^3). % of killing was determined by an *in vitro* cytotoxicity assay (72 hrs, n=3-5/group). n.s., one-way ANOVA with Tukey test.

(**B**) SKOV3 cells (2×10^4) were cocultured with 2×10^3 MSLN CAR-T$^{100\%MYXV}$ (100% MSLN CAR-T^{MYXV}), MSLN CAR-T$^{80\%MYXV}$ (80% MSLN CAR-T^{MYXV}+20% MSLN CAR-T), MSLN CAR-T$^{60\%MYXV}$ (60% MSLN CAR-T^{MYXV}+40% MSLN CAR-T), MSLN CAR-T$^{40\%MYXV}$ (40% MSLN CAR-T^{MYXV}+60% MSLN CAR-T), MSLN CAR-T$^{20\%MYXV}$ (20% MSLN CAR-T^{MYXV}+80% MSLN CAR-T), MSLN CAR-T$^{10\%MYXV}$ (10% MSLN CAR-T^{MYXV}+90% MSLN CAR-T), MSLN CAR-T$^{5\%MYXV}$ (5% MSLN CAR-T^{MYXV}+95% MSLN CAR-T), or MSLN CAR-T$^{1\%MYXV}$ (1% MSLN CAR-T^{MYXV}+99% MSLN CAR-T) cells. % of killing was determined by an *in vitro* cytotoxicity assay (72 hrs, n=5/group). ***P*<0.01, compared with MSLN CAR-T$^{100\%MYXV}$, one-way ANOVA with Tukey test.

(**C**) SKOV3 cells (2×10^4) were treated with MSLN CAR-T cells (2×10^3), MYXV (6×10^4 FFUs), MSLN CAR-T^{MYXV} cells (2×10^3), a mixture of 90% MSLN CAR-T (1.8×10^3) and 10% MSLN CAR-T^{MYXV} cells (0.2×10^3), CD19 CAR-T cells (2×10^3), a mixture of 90% CD19 CAR-T (1.8×10^3) and 10% CD19 CAR-T^{MYXV} cells (0.2×10^3), a mixture of 90% MSLN CAR-T (1.8×10^3) and 10% CD19 CAR-T^{MYXV} cells (0.2×10^3), or a mixture of 90% CD19 CAR-T (1.8×10^3) and 10% MSLN CAR-T^{MYXV} cells (0.2×10^3). % of killing was determined by an *in vitro* cytotoxicity assay (72 hrs, n=5/group). ***P*<0.01, 90% MSLN CAR-T+10% MSLN CAR-T^{MYXV} compared with any other groups, one-way ANOVA with Tukey test.

(**D**) hMSLN-expressing monocytes, hMSLN-expressing human umbilical vein endothelial cells (HUVECs), hMSLN-expressing epithelial (REPE-1) cells, and hMSLN-expressing stromal (HS-5) cells were generated by transduction with expression plasmid encoding hMSLN (monocytes^{MSLN}, HUVECs^{MSLN}, RWPE-1^{MSLN}, and HS-5^{MSLN}). SKOV3 cells, monocytes and monocytes^{MSLN}, HUVECs and HUVECs^{MSLN}, RWPE-1 and RWPE-1^{MSLN}, HS-5 and HS-5^{MSLN} (1×10^4) cells were treated with MYXV-tdTomato (3×10^4 FFUs) or MSLN CAR-T$^{MYXV\text{-}tdTomato}$ cells (1×10^3). % of tdTomato$^+$ cells was detected after 48 hrs (n=3-5/group). ***P*<0.01, SKOV3 compared with any other groups, one-way ANOVA with Tukey test.

(**E**) Monocytes and monocytes^{MSLN}, HUVECs and HUVECs^{MSLN}, RWPE-1 and RWPE-1^{MSLN}, HS-5 and HS-5^{MSLN} cells (1×10^4) were treated with MYXV (3×10^4 FFUs), MSLN CAR-T (1×10^3), MSLN CAR-T$^{10\%MYXV}$ (1×10^3), or MSLN CAR-T^{MYXV} cells (1×10^3). % of killing was determined by an *in vitro* cytotoxicity assay (72 hrs, n=3-5/group). n.s., MSLN CAR-T compared with MSLN CAR-T$^{10\%MYXV}$, one-way ANOVA with Tukey test.

(**F**) NSG mice bearing 40-day established SKOV3 tumors (1×10^7), indicated treatments were given when tumors reached ~9×8 mm on day 40. In group 3, the combination of MSLN CAR-T cells (2.5×10^6) and MYXV (2×10^7 FFUs) were i.v. injected. Survival curves are shown (n=10-12/group). n.s., MSLN CAR-T compared with MYXV (i.v.)+MSLN CAR-T, survival analysis was conducted by log-rank test.

(**G**) NSG mice bearing 40-day established SKOV3 tumors (1×10^7). Indicated treatments were given when tumors reached ~9×8 mm on day 40. Survival curves are shown (n=10-12/group). ***P*<0.01, compared with any other groups, survival analysis was conducted by log-rank test.

(**H**-**I**) Mice were euthanized on day 5, day 10, or day 15 after treatments as shown in schema in Figure 2C. (**H**) CD3$^+$ T cells were isolated from tumors and absolute CD3$^+$ T cell # in about 300 mg tumor is shown (n=4-5). **P*<0.05, ***P*<0.01, ****P*<0.001, compared with any other three groups, two-way ANOVA with posthoc Holm-Sidak test. (**I**) % of tdTomato$^+$ MSLN CAR-T^{MYXV} cells (% of tdTomato$^+$ CD3$^+$ T cells) is shown. **P*<0.05, Day 15 compared with any other groups, one-way ANOVA with Tukey test. Pooled results represent 2 independent experiments.

(**J**) MYXV replication (tdTomato$^+$ MYXV) in tumors was determined following indicated treatments. Treatment schema is shown in Figure 2C. Mice were euthanized on day 5, day 10, day 15, or day 25 after treatments (n=4-5). Total tumor (CD3$^+$ T cell-depleted) RNA was extracted, and *tdTomato* mRNA expression levels were analyzed by qPCR (n=3/group). ***P*<0.01, MYXV-tdTomato (i.t.)+MSLN CAR-T (Right) compared with any other groups, one-way ANOVA with Tukey test.

(**K**) Mixed 90% CD19 CAR-T (4.5×10^6) and 10% CD19 CAR-T^{MYXV} cells (0.5×10^6), mixed 90% MSLN CAR-T (4.5×10^6) and 10% CD19 CAR-T^{MYXV} cells (0.5×10^6), 100% MSLN CAR-T^{MYXV} cells (5×10^6), or mixed 90% MSLN CAR-T (4.5×10^6) and 10% MSLN CAR-T^{MYXV} cells (0.5×10^6) were transferred i.v. into NSG mice when SKOV3 tumors reached ~9×8 mm (1×10^7 SKOV3 cells challenged s.c. 40 days before ACT). Survival curves are shown (n=9-12/group). ****P*<0.001, compared with any other groups, survival analysis was conducted by log-rank test.

(**L**) Treatment schema is shown in Figure 2F. Mice were euthanized on day 8, day 16, or day 24 after treatments. CD3$^+$ T cells were isolated from tumors and absolute CD3$^+$ T cell # in about 300 mg tumor is shown (n=4-5). **P*<0.05, ***P*<0.01,

compared with any other three groups, two-way ANOVA with posthoc Holm-Sidak test. Pooled results represent 2 independent experiments.

(**M**) MYXV replication (tdTomato$^+$ MYXV) in tumors was determined following indicated treatments. Treatment schema is shown in Figure 2C. Mice were euthanized on day 8, day 16, day 24, or day 32 after treatments (n=4-5). A total tumor (CD3$^+$ T cell-depleted) RNA was extracted, and *tdTomato* mRNA expression levels were analyzed by qPCR (n=3/group). ***P*<0.01, MYXV-tdTomato (i.t.)+MART-1 T (Right) compared with any other groups, one-way ANOVA with Tukey test.

(**N**) Raji tumor-bearing NSG mice (i.v. established) received indicated treatments on day 10. In group 3, MYXV and CD19 CAR-T cells were i.v. injected. In groups 2 and 4, CD19 CAR-T cells or CD19 CAR-T$^{10\%MYXV}$ cells were i.v. injected. Survival curves are shown (n=10-12/group). ***P*<0.01, compared with any other groups, survival analysis was conducted by log-rank test.

(**A**-**E**, **H**-**J**, **L** and **M**) Data are mean ± SD.

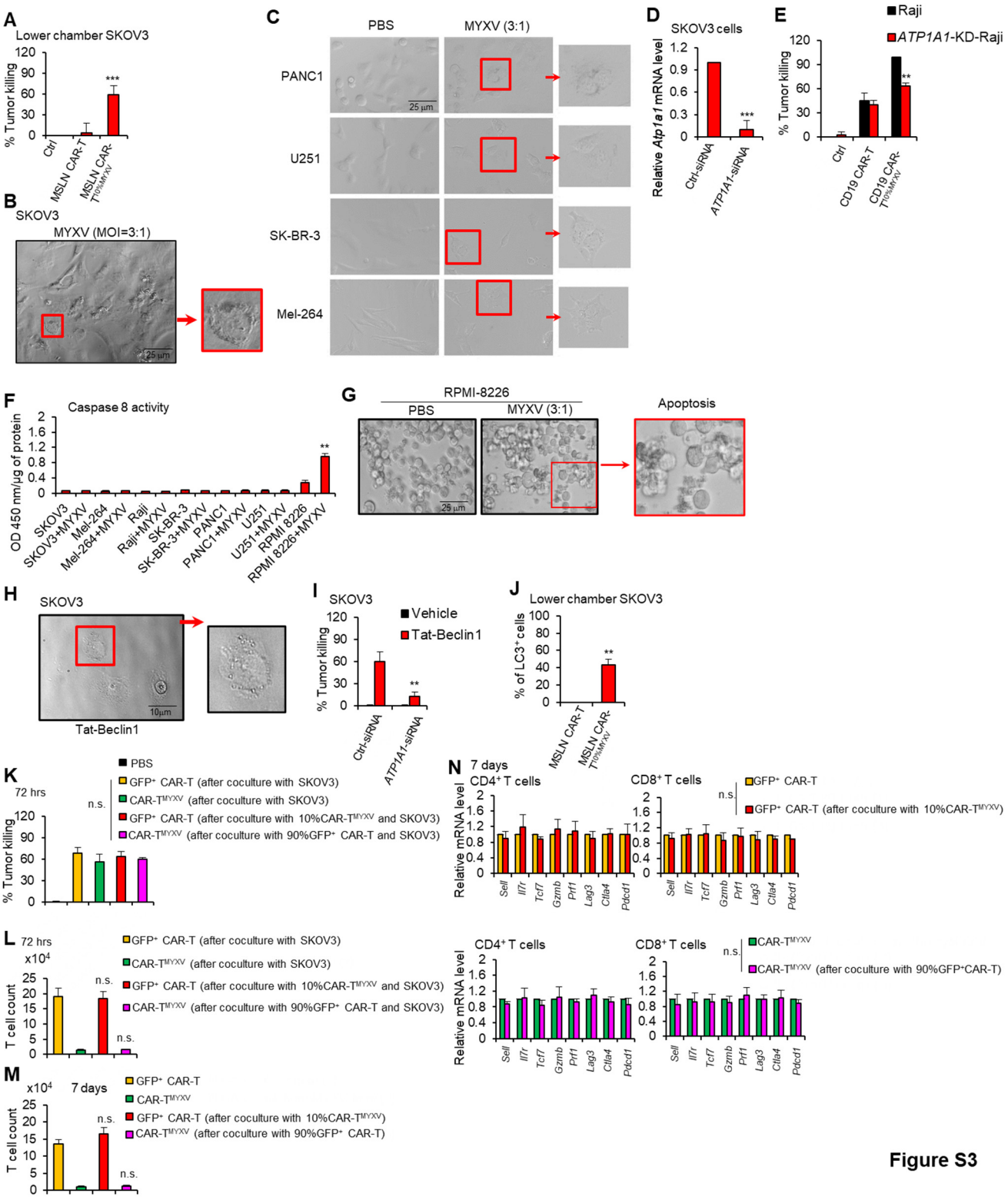
A
Lower chamber SKOV3
% Tumor killing
Ctrl
MSLN CAR-T
MSLN CAR-T10%MYXV
B
SKOV3
MYXV (MOI=3:1)
25 μm
C
PBS
MYXV (3:1)
PANC1
U251
SK-BR-3
Mel-264
D
SKOV3 cells
Relative Atp1a1 mRNA level
Ctrl-siRNA
ATP1A1-siRNA
E
Raji
ATP1A1-KD-Raji
% Tumor killing
Ctrl
CD19 CAR-T
CD19 CAR-T10%MYXV
F
Caspase 8 activity
OD 450 nm/μg of protein
SKOV3
SKOV3+MYXV
Mel-264
Mel-264+MYXV
Raji
Raji+MYXV
SK-BR-3
SK-BR-3+MYXV
PANC1
PANC1+MYXV
U251
U251+MYXV
RPMI 8226
RPMI 8226+MYXV
G
RPMI-8226
PBS
MYXV (3:1)
Apoptosis
H
SKOV3
10μm
Tat-Beclin1
I
SKOV3
Vehicle
Tat-Beclin1
% Tumor killing
Ctrl-siRNA
ATP1A1-siRNA
J
Lower chamber SKOV3
% of LC3+ cells
MSLN CAR-T
MSLN CAR-T10%MYXV
K
72 hrs
% Tumor killing
n.s.
PBS
GFP+ CAR-T (after coculture with SKOV3)
CAR-TMYXV (after coculture with SKOV3)
GFP+ CAR-T (after coculture with 10%CAR-TMYXV and SKOV3)
CAR-TMYXV (after coculture with 90%GFP+ CAR-T and SKOV3)
L
72 hrs
x10^4
T cell count
n.s.
GFP+ CAR-T (after coculture with SKOV3)
CAR-TMYXV (after coculture with SKOV3)
GFP+ CAR-T (after coculture with 10%CAR-TMYXV and SKOV3)
CAR-TMYXV (after coculture with 90%GFP+ CAR-T and SKOV3)
M
x10^4
7 days
T cell count
n.s.
GFP+ CAR-T
CAR-TMYXV
GFP+ CAR-T (after coculture with 10%CAR-TMYXV)
CAR-TMYXV (after coculture with 90%GFP+ CAR-T)
N
7 days
CD4+ T cells
CD8+ T cells
Relative mRNA level
Sell
Il7r
Tcf7
Gzmb
Prf1
Lag3
Ctla4
Pdcd1
n.s.
GFP+ CAR-T
GFP+ CAR-T (after coculture with 10%CAR-TMYXV)
CAR-TMYXV
CAR-TMYXV (after coculture with 90%GFP+CAR-T)

Figure S3

Figure S3. The role of MYXV and MYXV-loaded CAR-T cells on tumor cells. Related to Figure 3.

(**A**) SKOV3 cells were treated as shown in Figure 3B. % of killing was determined by an *in vitro* cytotoxicity assay (72 hrs, n=5/group). ***P<0.001, MSLN CAR-$T^{10\%MYXV}$ compared with any other groups, one-way ANOVA with Tukey test.

(**B-C**) SKOV3, PANC1 (human pancreatic ductal cell line), U251 (human glioblastoma cell line), SK-BR-3 (human breast cancer cell line), and Mel-264 cells (2×10^4) were treated with MYXV (6×10^4 FFUs) at MOI of 3:1 for 72 hrs. Representative images for the morphology of tumor cells are shown. Cell death (autosis) with a strong attachment to the culture plate is indicated with a red arrow. Scale bar, 25 μm.

(**D**) Control (Ctrl) or *ATP1A1*siRNA was transfected into SKOV3 cells for 72 hrs. Total RNA was extracted, and *Atp1a1* mRNA expression levels were analyzed by qPCR (n=3/group). ***P<0.001, Student's *t*-test.

(**E**) Control (Ctrl) or *ATP1A1* siRNA was transfected into Raji cells 72 hrs before further treatments. CD19 CAR-T or CD19 CAR-$T^{10\%MYXV}$ cells (2×10^3) were seeded to Raji and *ATP1A1*-KD Raji cells (2×10^4). After 72 hrs of coculture, % of killing was determined by an *in vitro* cytotoxicity assay (n=5). **P<0.01, two-way ANOVA with posthoc Holm-Sidak test.

(**F-G**) SKOV3, Mel-264, Raji, SK-BR-3, PANC1, U251, and RPMI-8226 cells (2×10^4) were treated with MYXV (6×10^4 FFUs) at MOI of 3:1 for 72 hrs. (**F**) Caspase 8 activity was measured by ELISA. **P<0.01, RPMI 8226+MYXV compared with RPMI-8226, one-way ANOVA with Tukey test. (**G**) Representative images (scale bar, 25 μm) for the morphology of RPMI-8226 cells are shown. Apoptotic cells are indicated with a red arrow.

(**H**) Tat-Beclin1 (10 μM) was coincubated with SKOV3 cells for 1.5 hrs. Representative images (scale bar, 10 μm) for the morphology of SKOV3 cells are shown. Cell death (autosis) with a strong attachment to the culture plate is indicated with a red arrow.

(**I**) Control (Ctrl) or *ATP1A1* siRNA was transfected into SKOV3 cells 72 hrs before further treatments. Tat-Beclin1 (10 μM) was coincubated with SKOV3 cells for 1.5 hrs. % of killing was determined by an *in vitro* cytotoxicity assay (n=5). **P<0.01, Tat-Beclin1 treated *ATP1A1*-KD SKOV3 cells compared with Tat-Beclin1 treated Ctrl-KD SKOV3 cells, two-way ANOVA with posthoc Holm-Sidak test.

(**J**) SKOV3 cells were treated as shown in Figure 3B. % of $LC3^+$ cells were detected by immunofluorescence. **P<0.01, Student's *t*-test.

(**K-L**) GFP^+ MSLN CAR-T cells, MSLN CAR-T^{MYXV}, or combined 90% GFP^+ MSLN CAR-T cells and 10% MSLN CAR-T^{MYXV} cells were cocultured with SKOV3 cells. GFP^+ MSLN CAR-T cells and MSLN CAR-T^{MYXV} were isolated from coculture after 72 hrs. (**K**) Isolated GFP^+ MSLN CAR-T and MSLN CAR-T^{MYXV} cells were used for cytotoxicity assay against SKOV3 cells. % of killing was determined by an *in vitro* cytotoxicity assay (72 hrs; n=5/group). n.s., one-way ANOVA with Tukey test. (**L**) Absolute T cell # is shown on 72 hrs (n=4-5). n.s., GFP^+ CAR-T (after coculture with 10%CAR-T^{MYXV} and SKOV3) compared with GFP^+ CAR-T (after coculture with 10%CAR-T^{MYXV} and SKOV3), CAR-T^{MYXV} (after coculture with 90%GFP^+ CAR-T and SKOV3) compared with CAR-T^{MYXV} (after coculture with SKOV3), one-way ANOVA with Tukey test.

(**M-N**) GFP^+ MSLN CAR-T cells, MSLN CAR-T^{MYXV}, or combined 90% GFP^+ MSLN CAR-T cells and 10% MSLN CAR-T^{MYXV} cells were cultured for 7 days. GFP^+ MSLN CAR-T cells and MSLN CAR-T^{MYXV} were isolated from coculture after 7 days. (**M**) Absolute T cell # is shown on day 7 (n=4-5). (**N**) $CD4^+$ T and $CD8^+$ T cells were sorted from isolated GFP^+ MSLN CAR-T and MSLN CAR-T^{MYXV} cells and extracted for total RNA. *Sell*, *Il7r*, *Tcf7*, *Gzmb*, *Prf1*, *Lag3*, *Ctla4,* and *Pdcd1* mRNA expression levels were analyzed by qPCR (n=3/group). n.s., GFP^+ CAR-T (after coculture with 10%CAR-T^{MYXV}) compared with GFP^+ CAR, CAR-T^{MYXV} (after coculture with 90%GFP^+ CAR-T) compared with CAR-T^{MYXV}, one-way ANOVA with Tukey test, one-way ANOVA with Tukey test.

(**A**, **D-F**, **I-N**) Data are mean ± SD.

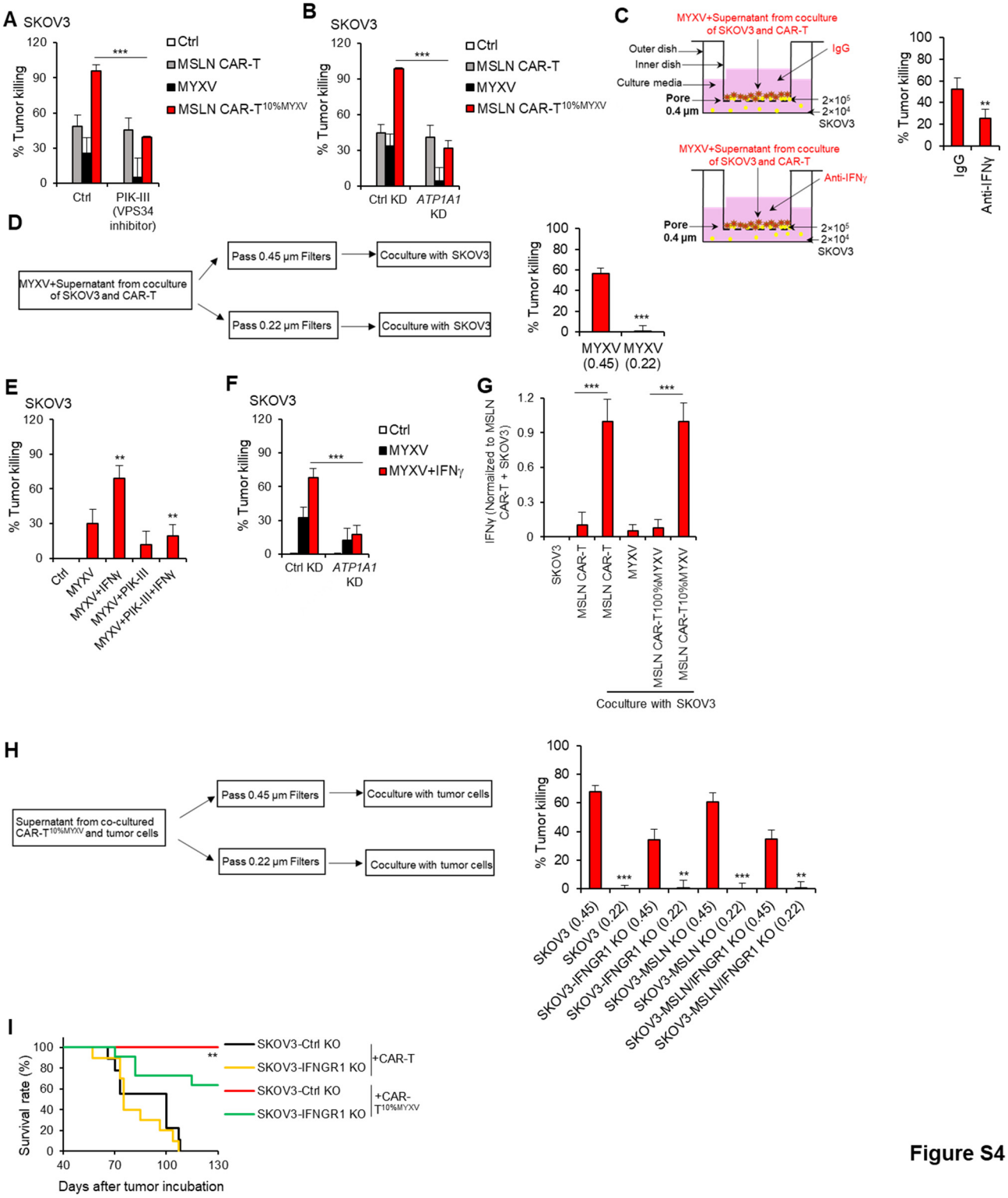
A
SKOV3
% Tumor killing
Ctrl
MSLN CAR-T
MYXV
MSLN CAR-T10%MYXV
Ctrl
PIK-III (VPS34 inhibitor)
B
SKOV3
% Tumor killing
Ctrl
MSLN CAR-T
MYXV
MSLN CAR-T10%MYXV
Ctrl KD
ATP1A1 KD
C
MYXV+Supernatant from coculture of SKOV3 and CAR-T
Outer dish
Inner dish
Culture media
Pore 0.4 μm
IgG
2×10^5
2×10^4
SKOV3
MYXV+Supernatant from coculture of SKOV3 and CAR-T
Anti-IFNγ
Pore 0.4 μm
2×10^5
2×10^4
SKOV3
% Tumor killing
IgG
Anti-IFNγ
D
MYXV+Supernatant from coculture of SKOV3 and CAR-T
Pass 0.45 μm Filters
Coculture with SKOV3
Pass 0.22 μm Filters
Coculture with SKOV3
% Tumor killing
MYXV (0.45)
MYXV (0.22)
E
SKOV3
% Tumor killing
Ctrl
MYXV
MYXV+IFNγ
MYXV+PIK-III
MYXV+PIK-III+IFNγ
F
SKOV3
% Tumor killing
Ctrl
MYXV
MYXV+IFNγ
Ctrl KD
ATP1A1 KD
G
IFNγ (Normalized to MSLN CAR-T + SKOV3)
SKOV3
MSLN CAR-T
MSLN CAR-T
MYXV
MSLN CAR-T100%MYXV
MSLN CAR-T10%MYXV
Coculture with SKOV3
H
Supernatant from co-cultured CAR-T10%MYXV and tumor cells
Pass 0.45 μm Filters
Coculture with tumor cells
Pass 0.22 μm Filters
Coculture with tumor cells
% Tumor killing
SKOV3 (0.45)
SKOV3 (0.22)
SKOV3-IFNGR1 KO (0.45)
SKOV3-IFNGR1 KO (0.22)
SKOV3-MSLN KO (0.45)
SKOV3-MSLN KO (0.22)
SKOV3-MSLN/IFNGR1 KO (0.45)
SKOV3-MSLN/IFNGR1 KO (0.22)
I
Survival rate (%)
SKOV3-Ctrl KO
SKOV3-IFNGR1 KO
+CAR-T
SKOV3-Ctrl KO
SKOV3-IFNGR1 KO
+CAR-T10%MYXV
Days after tumor incubation

Figure S4

Figure S4. Effect of VPS34 and IFNγ. Related to Figure 4.

(**A**) SKOV3 cells were pretreated with Ctrl (vehicle) or PIK-III (VPS34 inhibitor; 100 nM) for 12 hrs. MSLN CAR-T cells (2×10^3), MYXV (6×10^4 FFUs), or MSLN CAR-$T^{10\%MYXV}$ cells (2×10^3) were then cocultured with pretreated SKOV3 cells. % of killing was determined by an *in vitro* cytotoxicity assay (72 hrs; n=5/group). ***P<0.001, PIK-III (VPS34 inhibitor) compared with Ctrl group (with the treatment of MSLN CAR-$T^{10\%MYXV}$), two-way ANOVA with posthoc Holm-Sidak test.

(**B**) Control (Ctrl) or *ATP1A1* siRNA was transfected into SKOV3 cells 72 hrs before further treatments. MSLN CAR-T cells (2×10^3), MYXV (6×10^4 FFUs), or MSLN CAR-$T^{10\%MYXV}$ cells (2×10^3) were seeded to SKOV3 or *ATP1A1*-KD SKOV3 cells. After 72 hrs of coculture, % of killing was determined by an *in vitro* cytotoxicity assay (n=5). ***P<0.001, two-way ANOVA with posthoc Holm-Sidak test.

(**C**) SKOV3 cells were seeded into the upper (2×10^5) and lower (2×10^4) Transwell chambers. MYXV (6×10^5 FFUs) and culture supernatant (from coculture of SKOV3 and MSLN CAR-T cells) were only added to upper chambers. IgG or anti-IFNγ mAb (10 μg/ml) was added to the upper chambers. % of killing was determined by an *in vitro* cytotoxicity assay (n=5). **P<0.01, Student's *t*-test.

(**D**) MYXV and culture supernatant (from coculture of SKOV3 and MSLN CAR-T cells), after filtration with 0.45 μm or 0.22 μm, were added into SKOV3 cells. % of killing was determined by an *in vitro* cytotoxicity assay (n=5). ***P<0.001, Student's *t*-test.

(**E**) SKOV3 cells (2×10^4) were treated with MYXV (6×10^4 FFUs), MYXV (6×10^4 FFUs) plus hIFNγ (10 ng/ml), PIK-III (VPS34 inhibitor; 100 nM), or PIK-III (100 nM) plus hIFNγ (10 ng/ml). % of killing was determined by an *in vitro* cytotoxicity assay (72 hrs; n=5/group). **P<0.01, one-way ANOVA with Tukey test.

(**F**) Control (Ctrl) or *ATP1A1* siRNA was transfected into SKOV3 cells 72 hrs before further treatments. MYXV (6×10^4 FFUs) or MYXV (6×10^4 FFUs) plus hIFNγ (10 ng/ml) were seeded to SKOV3 cells. After 72 hrs of coculture, % of killing was determined by an *in vitro* cytotoxicity assay (n=5). ***P<0.001, two-way ANOVA with posthoc Holm-Sidak test.

(**G**) SKOV3 cells (2×10^4) were treated with MSLN CAR-T cells (2×10^3), MYXV (6×10^4 FFUs), MSLN CAR-T^{MYXV} or MSLN CAR-$T^{10\%MYXV}$ cells (2×10^3) for 72 hrs. Supernatants were analyzed by ELISA for hIFNγ concentrations (n=3/group). ***P<0.001, one-way ANOVA with Tukey test.

(**H**) MYXV and culture supernatant (from coculture of SKOV3 and MSLN CAR-T cells), after filtration with 0.45 μm or 0.22 μm, were added into SKOV3 and gene-modified SKOV3 cells. % of killing was determined by an *in vitro* cytotoxicity assay (n=5). **P<0.01, ***P<0.001, 0.45 μm compared with 0.22 μm, one-way ANOVA with Tukey test.

(**I**) MSLN CAR-T or MSLN CAR-$T^{10\%MYXV}$ cells (2.5×10^6) were transferred i.v. into NSG mice bearing SKOV3 tumors when tumors reached ~9×8 mm (1×10^7 SKOV3-Ctrl KO cells or 1×10^7 SKOV3-IFNGR1-KO cells challenged s.c. 40 days before ACT). Survival curves from two independent studies are summarized (n=9-11/group). **P<0.01, compared with any other groups, survival analysis was conducted by log-rank test.

(**A**-**H**) Data are mean ± SD.

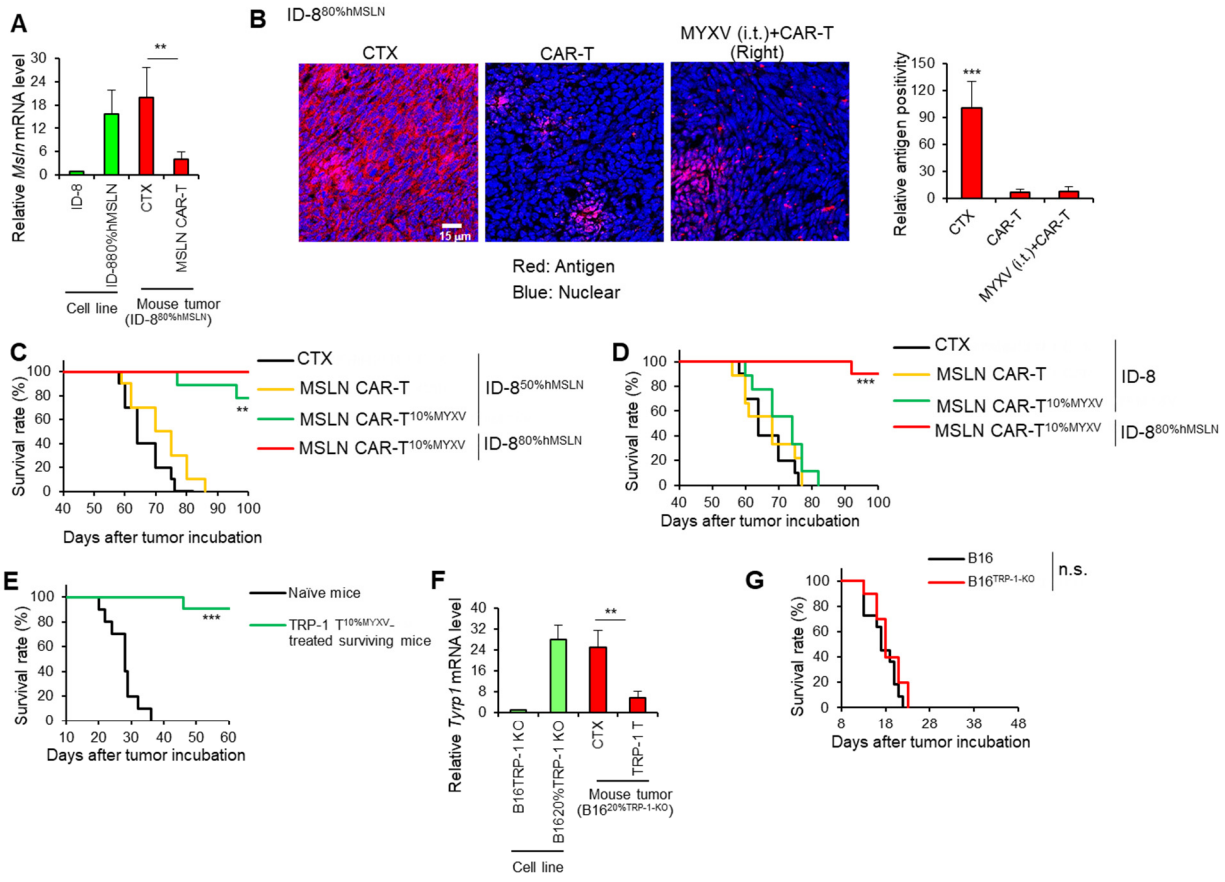
A
Relative Msln mRNA level
ID-8
ID-8^80%hMSLN
CTX
MSLN CAR-T
Cell line
Mouse tumor (ID-8^80%hMSLN)
B
ID-8^80%hMSLN
CTX
CAR-T
MYXV (i.t.)+CAR-T (Right)
15 μm
Red: Antigen
Blue: Nuclear
Relative antigen positivity
C
Survival rate (%)
Days after tumor incubation
CTX
MSLN CAR-T
MSLN CAR-T^10%MYXV
ID-8^50%hMSLN
MSLN CAR-T^10%MYXV
ID-8^80%hMSLN
D
Survival rate (%)
Days after tumor incubation
CTX
MSLN CAR-T
MSLN CAR-T^10%MYXV
ID-8
MSLN CAR-T^10%MYXV
ID-8^80%hMSLN
E
Survival rate (%)
Days after tumor incubation
Naïve mice
TRP-1 T^10%MYXV-treated surviving mice
F
Relative Tyrp1 mRNA level
B16TRP-1 KO
B16^20%TRP-1 KO
CTX
TRP-1 T
Cell line
Mouse tumor (B16^20%TRP-1-KO)
G
Survival rate (%)
Days after tumor incubation
B16
B16^TRP-1-KO
n.s.

Figure S5

Figure S5. Characterization of ID-8$^{80\%hMSLN}$ and B16$^{20\%TRP\text{-}1\text{-}KO}$ tumors. Related to Figure 5.

(**A**) Mice were treated as shown in Figure 5A. Tumor tissues from the CTX- and MSLN CAR-T cell-treated mice were harvested at the endpoint and extracted for total RNA. ID-8 cells and ID-8$^{80\%hMSLN}$ cells were also extracted for total RNA and used as controls. *hMSLN* mRNA expression levels were analyzed by qPCR (n=3/group). Data are mean ± SD. ***P*<0.01, MSLN CAR-T cells compared with CTX group, one-way ANOVA with Tukey test.

(**B**) Mice were treated as shown in Figure 5A. Tumor tissues from the CTX-, MSLN CAR-T cell- and MYXV (i.t.)+MSLN CAR-T cell-treated mice were harvested at the endpoint. Antigen expression levels were analyzed by IMC (n=3/group) and summarized data are shown (n=3/group). Scale bar, 15 μm. Data are mean ± SD. ****P*<0.001, CTX compared with any other groups, one-way ANOVA with Tukey test.

(**C**) MSLN CAR-T$^{10\%MYXV}$ cells were i.v. injected to B6 mice bearing s.c. established ID-8$^{50\%hMSLN}$ tumors (containing 50% WT ID-8 cells as ALVs) or ID-8$^{80\%hMSLN}$ tumors (containing 20% WT ID-8 cells as ALVs) on day 43 when tumors reached ~9×7 mm. Survival curves from two independent studies are summarized (n=8-10/group). ***P*<0.01, MSLN CAR-T$^{10\%MYXV}$ compared with MSLN CAR-T (ID-8$^{50\%hMSLN}$ tumors), survival analysis was conducted by log-rank test.

(**D**) MSLN CAR-T$^{10\%MYXV}$ cells were i.v. injected to B6 mice bearing s.c. established ID-8 tumors (100% WT ID-8 cells) or ID-8$^{80\%hMSLN}$ tumors on day 43 when tumors reached ~9×7 mm. Survival curves from two independent studies are summarized (n=8-10/group). ****P*<0.001, MSLN CAR-T$^{10\%MYXV}$ (ID-8$^{80\%hMSLN}$ tumors) compared with any other groups, survival analysis was conducted by log-rank test.

(**E**) B16$^{20\%TRP\text{-}1\text{-}KO}$ tumor (containing 20% B16 TRP-1-KO ALVs)-bearing mice were treated similar to Figure 5D. Surviving mice from the TRP-1 T$^{10\%MYXV}$-treated group were re-challenged with 3×10^5 ALVs (B16$^{TRP\text{-}1\text{-}KO}$) on day 60. Survival curves from two independent studies are summarized (n=8-10/group). ****P*<0.001, survival analysis was conducted by log-rank test.

(**F**) Mice were treated as shown in Figure 5D. Tumor tissues from the CTX- and TRP-1 T cell-treated mice were harvested at the endpoint and extracted for total RNA. B16$^{TRP\text{-}1\text{-}KO}$ cells and B16$^{20\%TRP\text{-}1\text{-}KO}$ cells were also extracted for total RNA and used as controls. *Tyrp1* (gene encoding TRP-1) mRNA expression levels were analyzed by qPCR (n=3/group). Data are mean ± SD. ***P*<0.01, TRP-1 T cells compared with CTX group, one-way ANOVA with Tukey test.

(**G**) B6 mice were inoculated s.c with B16 or B16$^{20\%TRP\text{-}1\text{-}KO}$ cells (1×10^6) without receiving any treatments. Survival curves are shown (n=10-12/group). n.s., survival analysis was conducted by log-rank test.

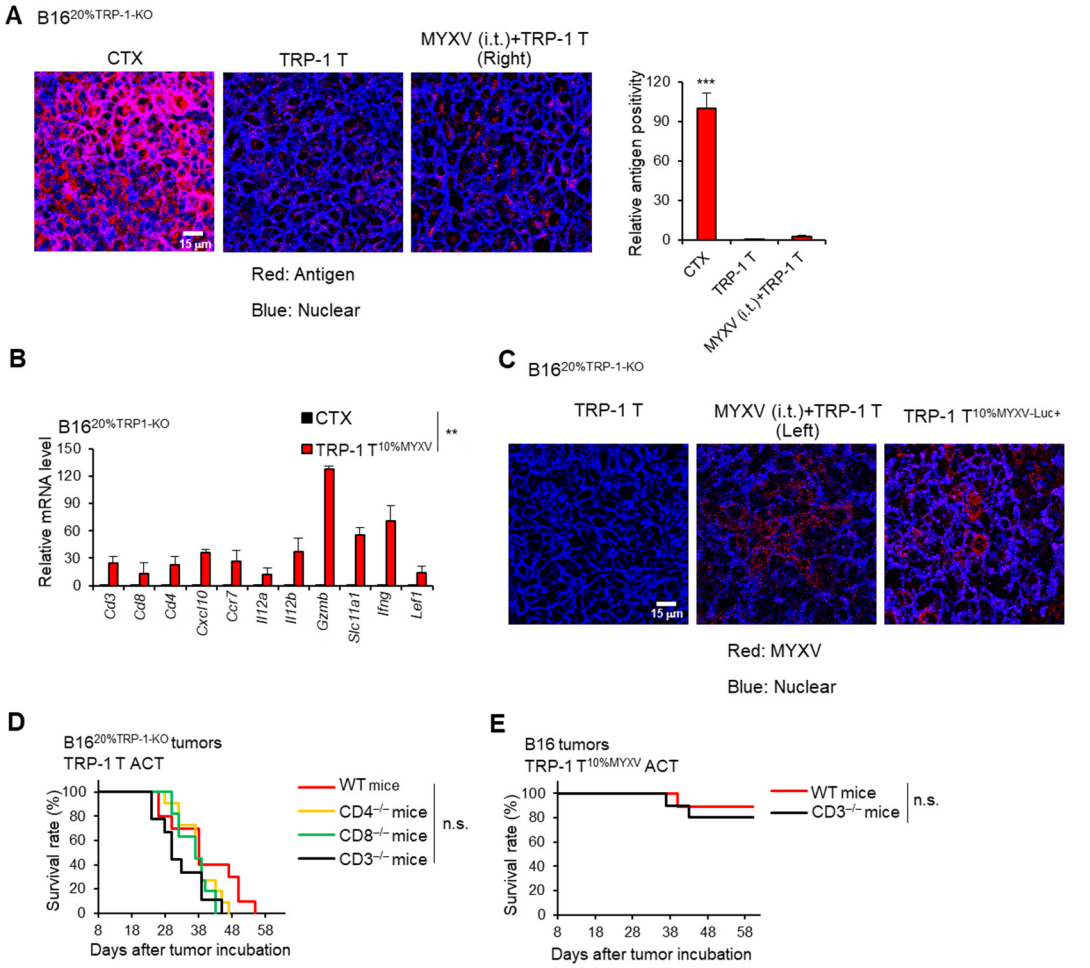
A
B16 20%TRP-1-KO
CTX
TRP-1 T
MYXV (i.t.)+TRP-1 T (Right)
15 μm
Red: Antigen
Blue: Nuclear
Relative antigen positivity
120
90
60
30
0
***
CTX
TRP-1 T
MYXV (i.t.)+TRP-1 T
B
B16 20%TRP1-KO
CTX
TRP-1 T 10%MYXV
**
Relative mRNA level
150
120
90
60
30
0
Cd3
Cd8
Cd4
Cxcl10
Ccr7
Il12a
Il12b
Gzmb
Slc11a1
Ifng
Lef1
C
B16 20%TRP-1-KO
TRP-1 T
MYXV (i.t.)+TRP-1 T (Left)
TRP-1 T 10%MYXV-Luc+
15 μm
Red: MYXV
Blue: Nuclear
D
B16 20%TRP-1-KO tumors
TRP-1 T ACT
Survival rate (%)
100
80
60
40
20
0
8
18
28
38
48
58
Days after tumor incubation
WT mice
CD4−/− mice
CD8−/− mice
CD3−/− mice
n.s.
E
B16 tumors
TRP-1 T 10%MYXV ACT
Survival rate (%)
100
80
60
40
20
0
8
18
28
38
48
58
Days after tumor incubation
WT mice
CD3−/− mice
n.s.

Figure S6

Figure S6. Effect of host immune cells. Related to Figure 6.

(**A**) Mice were treated as shown in Figure 5D. Tumor tissues from the CTX-, TRP-1 T cell- and MYXV (i.t.)+TRP-1 T cell-treated mice were harvested at the endpoint. Antigen expression levels were analyzed by IMC (n=3/group) and summarized data are shown (n=3/group). Scale bar, 15 μm. Data are mean ± SD. ***P<0.001, CTX compared with any other groups, one-way ANOVA with Tukey test.

(**B**) Mice were treated as shown in Figure 6A**.** Tumor tissues from the CTX- and TRP-1 $T^{10\%MYXV}$ cell-treated mice were harvested at the endpoint and extracted for total RNA. *Cd3, Cd8, Cd4, Cxcl10, Ccr7, Il12a, Il12b, Gzmb, Slc11a1, Ifng, and Lef1* mRNA expression levels were analyzed by qPCR (n=3/group). Data are mean ± SD. **P<0.01, TRP-1 $T^{10\%MYXV}$ cell-treated group compared with CTX-treated group, one-way ANOVA with Tukey test.

(**C**) Mice were treated as shown in Figure 6A. Tumor tissues from the TRP-1 T, MYXV-Luc^+ (L. i.t.) +TRP-1 (Left) and TRP-1 $T^{10\%MYXV\text{-}Luc^+}$-treated mice were harvested on day 10 after treatments. MYXV infection was analyzed by IMC (n=3/group). Scale bar, 15 μm.

(**D**) TRP-1 T cells (5×10^6) were transferred i.v. into WT, $CD4^{-/-}$, $CD8^{-/-}$, and $CD3^{-/-}$ B6 mice when $B16^{20\%TRP\text{-}1\text{-}KO}$ tumors reached ~7×6 mm (1×10^6 $B16^{20\%TRP\text{-}1\text{-}KO}$ challenged s.c. 8 days before ACT; adjuvant CTX was administered i.p. to mice one day before ACT). Survival curves are shown (n=9-11/group). n.s., survival analysis was conducted by log-rank test.

(**E**) WT and $CD3^{-/-}$ B6 mice were s.c. inoculated with B16 tumor cells (1×10^6). TRP-1 $T^{10\%MYXV}$ cells (5×10^6) were transferred i.v. into mice when tumors reached ~7×6 mm on day 8. Adjuvant CTX was administered i.p. to mice one day before ACT. Survival curves are shown (n=9-11/group). n.s., survival analysis was conducted by log-rank test.