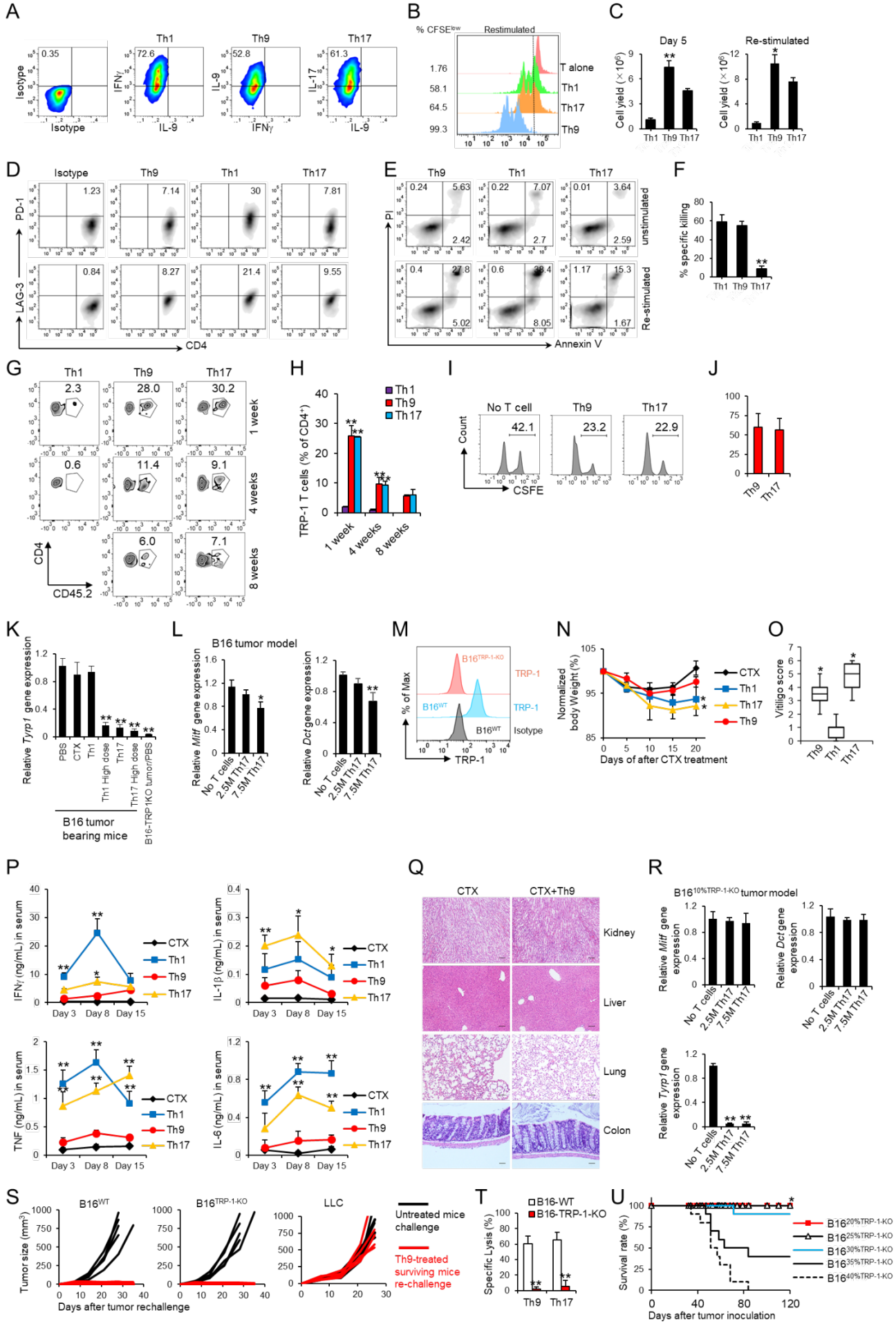

A
Th1
Th9
Th17
Isotype
IFNγ
IL-9
IL-17
0.35
72.6
52.8
61.3
B
% CFSE^low
Restimulated
T alone
Th1
Th17
Th9
1.76
58.1
64.5
99.3
C
Day 5
Re-stimulated
Cell yield (×10^6)
Th1 Th9 Th17
D
Isotype
Th9
Th1
Th17
PD-1
LAG-3
CD4
1.23
7.14
30
7.81
0.84
8.27
21.4
9.55
E
Th9
Th1
Th17
PI
Annexin V
unstimulated
Re-stimulated
0.24
5.63
2.42
0.22
7.07
2.7
0.01
3.64
2.59
0.4
27.8
5.02
0.6
38.4
8.05
1.17
15.3
1.67
F
% specific killing
Th1 Th9 Th17
G
Th1
Th9
Th17
1 week
4 weeks
8 weeks
CD4
CD45.2
2.3
28.0
30.2
0.6
11.4
9.1
6.0
7.1
H
Th1
Th9
Th17
TRP-1 T cells (% of CD4+)
1 week
4 weeks
8 weeks
I
No T cell
Th9
Th17
Count
CSFE
42.1
23.2
22.9
J
Th9
Th17
K
Relative Tyrp1 gene expression
PBS
CTX
Th1
Th1 High dose
Th17
Th17 High dose
B16-TRP1KO tumor/PBS
B16 tumor bearing mice
L
B16 tumor model
Relative Mitf gene expression
Relative Dct gene expression
No T cells
2.5M Th17
7.5M Th17
M
B16^TRP-1-KO
B16^WT
TRP-1
Isotype
% of Max
TRP-1
N
Normalized body Weight (%)
Days of after CTX treatment
CTX
Th1
Th17
Th9
O
Vitiligo score
Th9
Th1
Th17
P
IFNγ (ng/mL) in serum
IL-1β (ng/mL) in serum
TNF (ng/mL) in serum
IL-6 (ng/mL) in serum
Day 3
Day 8
Day 15
CTX
Th1
Th9
Th17
Q
CTX
CTX+Th9
Kidney
Liver
Lung
Colon
R
B16^10%TRP-1-KO tumor model
Relative Mitf gene expression
Relative Dct gene expression
Relative Tyrp1 gene expression
No T cells
2.5M Th17
7.5M Th17
S
B16^WT
B16^TRP-1-KO
LLC
Tumor size (mm^3)
Days after tumor rechallenge
Untreated mice challenge
Th9-treated surviving mice re-challenge
T
B16-WT
B16-TRP-1-KO
Specific Lysis (%)
Th9
Th17
U
Survival rate (%)
Days after tumor inoculation
B16^20%TRP-1-KO
B16^25%TRP-1-KO
B16^30%TRP-1-KO
B16^35%TRP-1-KO
B16^40%TRP-1-KO

Figure S1, related to Figure 1. Basic properties of murine Th1, Th9, and Th17 cells after polarization *in vitro*; and persistence, cytolytic function and toxicity assessment of transferred murine Th1, Th9, and Th17 cells *in vivo*.

(**A**) Naïve $CD4^+CD62L^+$ T cells were purified from the spleens of TRP-1 mice and cocultured with irradiated APCs under polarized conditions as detailed in the Methods. Intracellular staining showing the percentages of cytokine-producing cells in polarized Th1, Th9 and Th17 cells. Representative data are shown.

(**B**) TRP-1 Th cells (polarized in vitro for 5 days) were labeled with CFSE and cocultured with TRP-1 peptide-pulsed APCs (restimulated) for 48 hrs. T alone is Th cells fixed with paraformaldehyde immediately after CFSE labeling. The percentage of $CFSE^{low}$ proliferative cells was determined by FACS. Representative data are shown.

(**C**) Th cell yields after the first round of activation (day 5, the initial T cell number was 3×10^5) and after restimulation by TRP-1 peptide-pulsed APCs for an additional 4 days (second round, 3×10^5 of cells of TRP-1-Th cells were collected for the restimulation) (n=3). Data are mean ± SD. $^*P<0.05$, $^{**}P<0.01$, compared with Th1 and Th17 groups, one-way ANOVA with Tukey test.

(**D**) Surface expression of indicated exhaustion markers after restimulation by TRP-1 peptide-pulsed APCs *in vitro* was determined by FACS.

(**E**) FACS analysis of apoptotic Th cells one day after restimulation with TRP-1 peptide-pulsed APCs *in vitro*.

(**F**) Specific killing assay of TRP-1 Th cells before transfer was performed against B16 cells (n = 4/group). T cell to tumor ratio = 10:1. Data are mean ± SD. $^{**}P<0.01$, compared with Th1 and Th17 groups, one-way ANOVA with Tukey test.

(**G-H**) TRP-1-specific Th1, Th17, or Th9 cells ($CD45.2^+$, 2.5×10^6) were transferred i.v. into $CD45.1^+$ B6 mice bearing 10-day established B16 tumors (1×10^6 B16 cells challenged s.c. 10 days before T cell transfer). Adjuvant cyclophosphamide (CTX, i.p.) and DC vaccination (2.5×10^5, i.v.) were administered to some mice as indicated in Fig. 1A. The transferred T cell-derived cells ($CD45.2^+CD4^+$) were detected from blood by FACS (n=3/goup). Representative data (**G**) and summarized results (**H**) are shown. Data are mean ± SD. $^{**}P<0.01$, compared with Th1 group, one-way ANOVA with Tukey test.

(**I-J**) *In vivo* cytotoxic T lymphocyte (CTL) assay. $CFSE^{lo}$ (left population out gates, non-target) and $CFSE^{hi}$ (right population in gates, target) cells were pulsed with control peptide and TRP-1 peptide, respectively, and were injected into mice ~50 days after ACT. Splenocytes were collected 24 h later for detection of CFSE-labelled cells. Results are representative of three independent experiments (**I**), and Pooled data (n=3) for *in vivo* CTL assay (**J**).

(**K-L**) Tumors were harvested at the endpoint from mice in Fig. 1B. Tumor tissues were analyzed by qPCR for *Tyrp1* (**K**), *Mitf*, and *Dct* (**L**) mRNA levels (n=3). Data are mean ± SD. $^{**}P<0.01$, compared with PBS, CTX, and Th1 groups (K), $^*P<0.05$, $^{**}P<0.01$, compared with No T cells group (L), one-way ANOVA with Tukey test.

(**M**) Intracellular staining of TRP-1 in $B16^{WT}$ and $B16^{TRP\text{-}1KO}$ tumor cells.

(**N**) Normalized follow-up of body weight of $B16^{10\%TRP\text{-}1\text{-}KO}$ tumor (s.c.)-bearing B6 mice treated with a regular dose of TRP-1 Th1, Th9, or Th17 cells similar to Fig. 1F (n=5/group). Data are mean ± SD. $^*P<0.05$, Th1 or Th17 compared with CTX group, one-way ANOVA with Tukey test.

(**O**) Summarized vitiligo score (n ⩾8) of $B16^{10\%TRP\text{-}1\text{-}KO}$ tumor (s.c.)-bearing mice treated with a regular dose of TRP-1 Th1, Th9, or Th17 cells (similar to Fig. 1F) 45 days after transfer or at the endpoint. Vitiligo score: 0 (no vitiligo); 1 (vitiligo detected); 2 (> 10% vitiligo); 3 (> 30% vitiligo); 4 (> 50% vitiligo); 5 (> 75% vitiligo); 6 (> 90% vitiligo). Data are mean ± SD. $^{*}P<0.05$, compared with Th1 group, one-way ANOVA with Tukey test.

(**P**) Cytokine levels in serum of $B16^{10\%TRP\text{-}1\text{-}KO}$ tumor (s.c.)-bearing mice treated with a regular dose of TRP-1 Th1, Th9, or Th17 cells similar to Fig. 1F (n=3). Data are mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, compared with Th9 group, one-way ANOVA with Tukey test.

(**Q**) $B16^{10\%TRP\text{-}1\text{-}KO}$ tumor (s.c.)-bearing mice treated with CTX or TRP-1 Th9 cells similar to Fig. 1F. Tissue pathology of the indicated organs was evaluated by H&E staining (CTX group at the endpoint; Th9 group on day 45). Scale bars, 100 μm. Representative data are shown.

(**R**) TRP-1-specific Th17 cells (regular dose, 2.5×10^6) or high dose Th17 (7.5×10^6) were transferred i.v. into B6 mice bearing 10-day established $B16^{10\%TRP\text{-}1\text{-}KO}$ tumors (1×10^6 $B16^{10\%TRP\text{-}1\text{-}KO}$ cells challenged s.c. 10 days before T cell transfer). Adjuvant cyclophosphamide (CTX, i.p.) and DC vaccination (2.5×10^5, i.v.) were administered to mice as indicated in Fig. 1A. Tumors were harvested at the endpoint from mice and tumor tissues were analyzed by qPCR for *Mitf*, *Dct*, and *Tyrp1* mRNA levels (n=3). Data are mean ± SD. $^{**}P<0.01$, compared with No T cells group, one-way ANOVA with Tukey test.

(**S**) Surviving mice from Fig. 1F were rechallenged with 2×10^6 indicated tumor cells at day 60 (s.c. injection on the contralateral flank). Tumor growth is shown (n=5/group).

(**T**) Mice treated as in Fig. 1F were euthanized and splenic transferred cell-derived cells ($CD45.2^+CD4^+$) were isolated for *in vitro* cytolytic assay (n=3/group). Data are mean ± SD. $^{**}P<0.01$, compared with B16-WT, one-way ANOVA with Tukey test.

(**U**) TRP-1-specific Th9 cells (2.5×10^6) were transferred i.v. into B6 mice bearing 10-day established B16 tumors containing 20%, 25%, 30%, 35%, or 40% of TRP-1-KO B16 tumor cells. Adjuvant cyclophosphamide (CTX, i.p.) and DC vaccination (2.5×10^5, i.v.) were administered to mice as indicated in Fig. 1A. Survival analysis in response to adoptive Th9 cell transfer (n=9-10/group, combined from 2 independent experiments). Data are summarized from two independent studies. $^{*}P<0.05$, 20%, 25%, or 30% compared with 35% or 40%, survival analysis was conducted by log-rank test.

Representative results from one of two repeated experiments are shown.

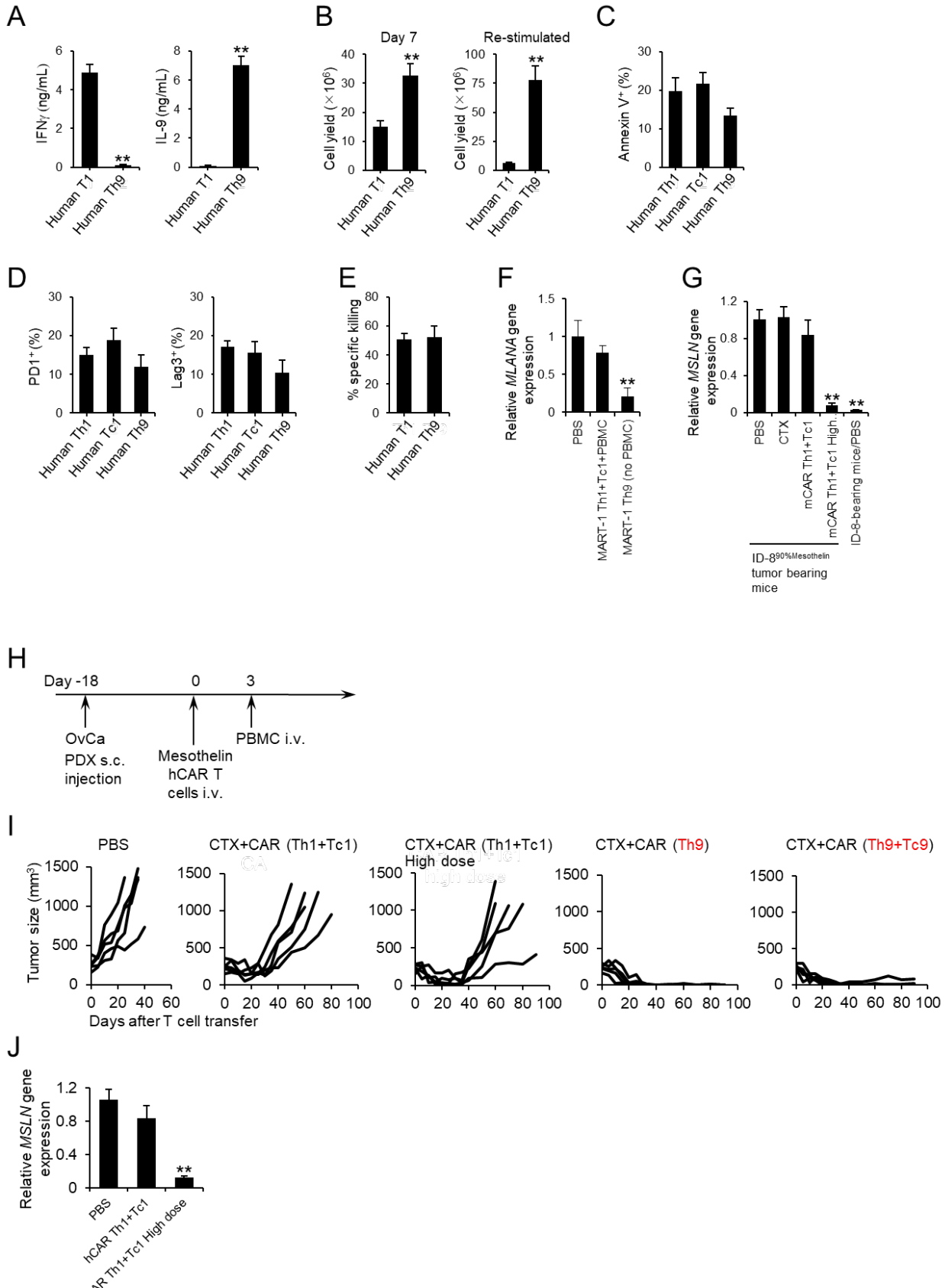

A
IFNγ (ng/mL)
IL-9 (ng/mL)
Human T1
Human Th9
B
Day 7
Re-stimulated
Cell yield (×10^6)
C
Annexin V+ (%)
Human Th1
Human Tc1
Human Th9
D
PD1+ (%)
Lag3+ (%)
E
% specific killing
F
Relative MLANA gene expression
PBS
MART-1 Th1+Tc1+PBMC
MART-1 Th9 (no PBMC)
G
Relative MSLN gene expression
CTX
mCAR Th1+Tc1
mCAR Th1+Tc1 High.
ID-8-bearing mice/PBS
ID-8 90%Mesothelin tumor bearing mice
H
Day -18
0
3
OvCa PDX s.c. injection
Mesothelin hCAR T cells i.v.
PBMC i.v.
I
PBS
CTX+CAR (Th1+Tc1)
CTX+CAR (Th1+Tc1) High dose
CTX+CAR (Th9)
CTX+CAR (Th9+Tc9)
Tumor size (mm3)
Days after T cell transfer
J
Relative MSLN gene expression
PBS
hCAR Th1+Tc1
hCAR Th1+Tc1 High dose

Figure S2, related to Figure 2. Properties and anti-tumor effect of human CAR T cells.

(**A**) Cytokines were measured by ELISA in the supernatants 4 days after the polarization of human T cells *in vitro* (n = 3). Data are mean ± SD. $^{**}P<0.01$, compared to human T1 group, Student's *t*-tests.

(**B**) Cell yields after the first round of activation (day 7, the initial cell number was 1×10^6, n = 3) and after restimulation for an additional 4 days (second round, 1×10^6 of cells were collected for the restimulation). Data are mean ± SD. $^{**}P<0.01$, compared to human T1 group, Student's *t*-tests.

(**C**-**D**) The percentage of apoptotic cells (**C**) and surface expression of indicated exhaustion markers (**D**) one day after restimulation with Dynabeads *in vitro* was determined by FACS (n=3).

(**E**) Specific killing assay of Mesothelin-targeting CAR-T1 and CAR-Th9 cells before transfer was performed against human SK-OV-3 tumor cells (n = 3/group). T cell to tumor ratio = 2:1.

(**F**) Tumors were harvested at the endpoint from mice in Fig. 2E. Tumor tissues were analyzed by qPCR for *MART1* mRNA levels (n=3). Data are mean ± SD. $^{**}P<0.01$, compared with PBS and Th1+Tc1 groups, one-way ANOVA with Tukey test.

(**G**) Tumors were harvested at the endpoint from mice in Fig. 2F. Tumor tissues were analyzed by qPCR for *MSLN* mRNA levels (n=3). Data are mean ± SD. $^{**}P<0.01$, compared with PBS, CTX, and Th1+Tc1 regular dose groups, one-way ANOVA with Tukey test.

(**H**) Human Mesothelin CAR T cells (5×10^6 CAR Th1+Tc1, 5×10^6 CAR Th9, 5×10^6 CAR Th9+Tc9, or high dose of 2.5×10^7 Th1+Tc1 cells) and autologous PBMC (1×10^7) were given to DKO-NSG mice.

(**I**) Tumor responses to T cell transfer are shown (n=5/group).

(**J**) Tumors were harvested from treated mice at the endpoints (PBS: ~day 20; Th1+Tc1-treated mice: ~day 50). Tumor tissues were analyzed by qPCR for *MSLN* mRNA levels (n=3). Data are mean ± SD. $^{**}P<0.01$, compared with PBS and Th1+Tc1 regular dose groups, one-way ANOVA with Tukey test.

Representative results from one of two repeated experiments are shown.

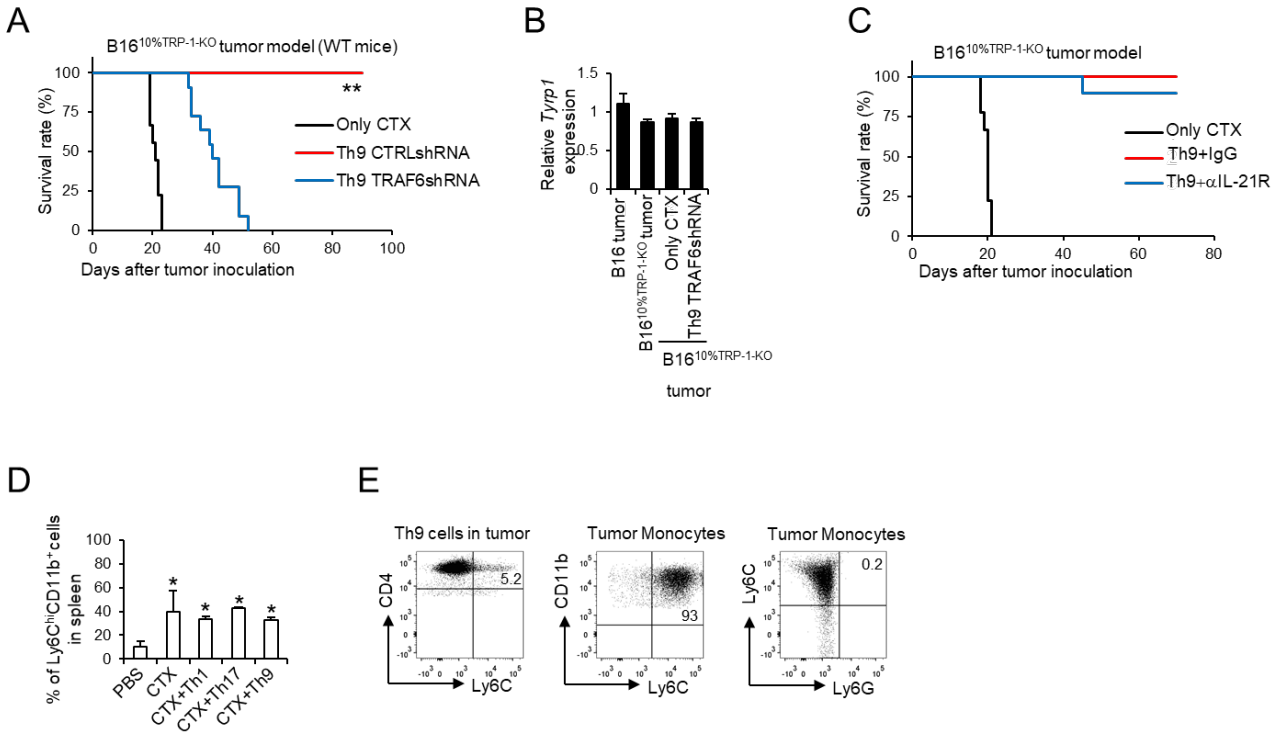


Figure S3, related to Figure 3. TRAF6 signaling in Th9 cells was required for the initial antitumor response and cells from the spleen or tumor were analyzed by FACS.

(**A**-**B**) TRP-1-specific Th9 cells (2.5×10^6) were transferred i.v. into WT mice bearing 10-day established $B16^{10\%TRP\text{-}1\text{-}KO}$ tumors (1×10^6 $B16^{10\%TRP\text{-}1\text{-}KO}$ cells challenged s.c. 10 days before T cell transfer). Adjuvant cyclophosphamide (CTX, i.p.) and DC vaccination (2.5×10^5, i.v.) were administered to mice as indicated in Fig. 1A. (**A**) Survival curves (n=9-12/group, combined from 2 independent experiments). $^{**}P<0.01$, compared with any other groups, survival analysis was conducted by log-rank test. (**B**) Tumors were harvested at the endpoint from mice and tumor tissues were analyzed by qPCR for *Tyrp1* mRNA levels (n=3).

(**C**) TRP-1-specific Th9 cells (2.5×10^6) were transferred i.v. into WT mice bearing 10-day established $B16^{10\%TRP\text{-}1\text{-}KO}$ tumors (1×10^6 $B16^{10\%TRP\text{-}1\text{-}KO}$ cells challenged s.c. 10 days before T cell transfer). Adjuvant cyclophosphamide (CTX, i.p.) and DC vaccination (2.5×10^5, i.v.) were administered to mice as indicated in Fig. 1A. IgG control or αIL-21R antibody (150 μg/mouse) was i.p injected every 3 days starting one day before ACT for 15 doses. Survival curves are shown (n=9-12/group, combined from 2 independent experiments). Survival analysis was conducted by log-rank test.

(**D**-**E**) Mice were treated as in Fig. 1F. (**D**) Splenic inflammatory monocytes were determined on day 10 after ACT by FACS (n=3-4/group). Data are mean ± SD. $^{*}P<0.05$, compared with PBS group, one-way ANOVA with Tukey test. (**E**) The surface expression of Ly6C on Th9 cells or CD11b cells in tumors with Th9 ACT was determined on day 10 after ACT by FACS.

Representative results from one of two repeated experiments are shown.

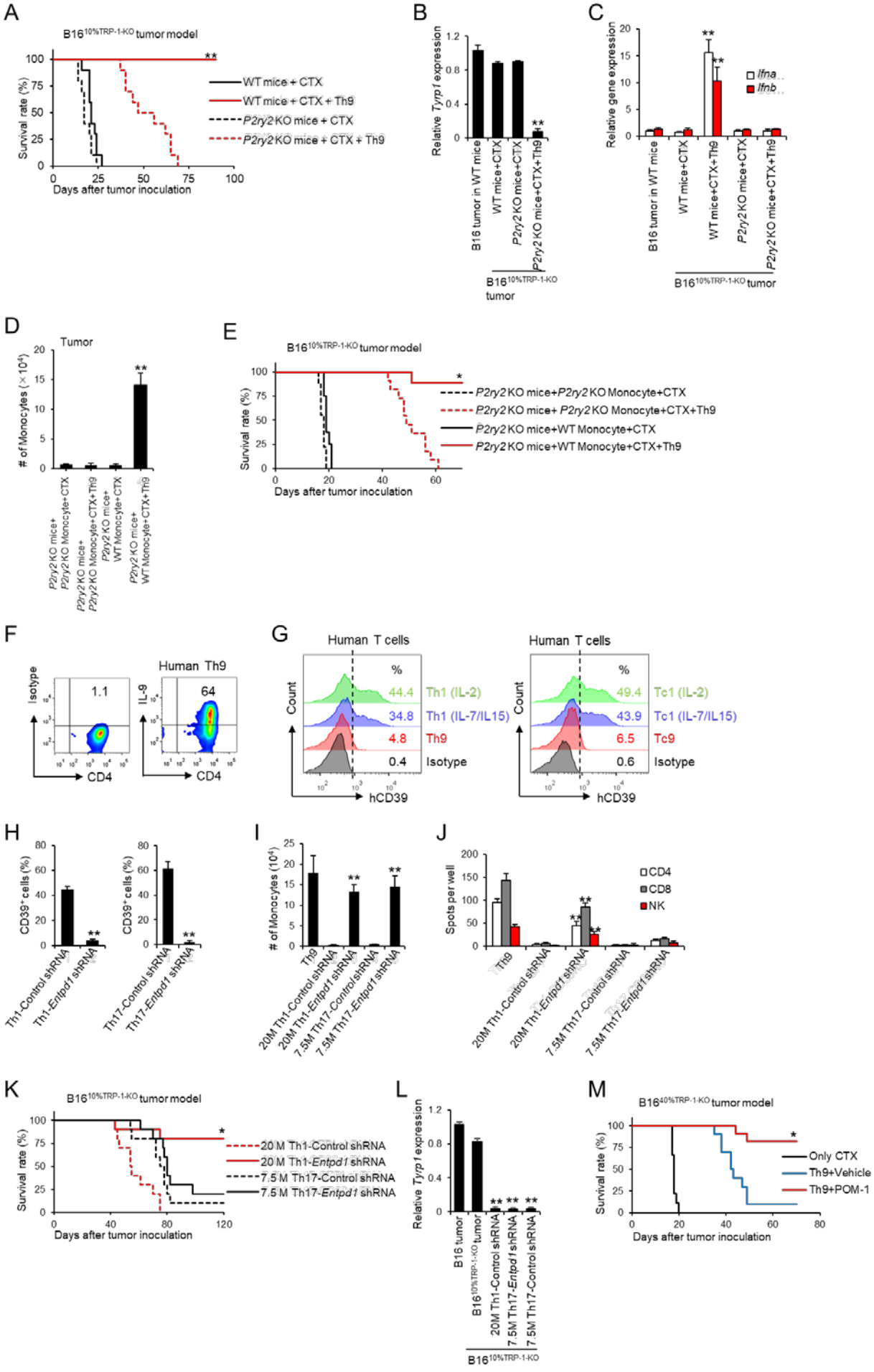

A
B16 10%TRP-1-KO tumor model
Survival rate (%)
Days after tumor inoculation
WT mice + CTX
WT mice + CTX + Th9
P2ry2 KO mice + CTX
P2ry2 KO mice + CTX + Th9
B
Relative Tyrp1 expression
B16 tumor in WT mice
WT mice+CTX
P2ry2 KO mice+CTX
P2ry2 KO mice+CTX+Th9
B16 10%TRP-1-KO tumor
C
Relative gene expression
Ifna
Ifnb
B16 tumor in WT mice
WT mice+CTX
WT mice+CTX+Th9
P2ry2 KO mice+CTX
P2ry2 KO mice+CTX+Th9
B16 10%TRP-1-KO tumor
D
Tumor
# of Monocytes (×10⁴)
P2ry2 KO mice+ P2ry2 KO Monocyte+CTX
P2ry2 KO mice+ P2ry2 KO Monocyte+CTX+Th9
P2ry2 KO mice+ WT Monocyte+CTX
P2ry2 KO mice+ WT Monocyte+CTX+Th9
E
B16 10%TRP-1-KO tumor model
Survival rate (%)
Days after tumor inoculation
P2ry2 KO mice+P2ry2 KO Monocyte+CTX
P2ry2 KO mice+ P2ry2 KO Monocyte+CTX+Th9
P2ry2 KO mice+WT Monocyte+CTX
P2ry2 KO mice+WT Monocyte+CTX+Th9
F
Human Th9
Isotype
IL-9
1.1
64
CD4
CD4
G
Human T cells
Human T cells
Count
%
44.4
Th1 (IL-2)
34.8
Th1 (IL-7/IL15)
4.8
Th9
0.4
Isotype
49.4
Tc1 (IL-2)
43.9
Tc1 (IL-7/IL15)
6.5
Tc9
0.6
Isotype
hCD39
hCD39
H
CD39⁺ cells (%)
CD39⁺ cells (%)
Th1-Control shRNA
Th1-Entpd1 shRNA
Th17-Control shRNA
Th17-Entpd1 shRNA
I
# of Monocytes (10⁴)
Th9
20M Th1-Control shRNA
20M Th1-Entpd1 shRNA
7.5M Th17-Control shRNA
7.5M Th17-Entpd1 shRNA
J
Spots per well
CD4
CD8
NK
Th9
20M Th1-Control shRNA
20M Th1-Entpd1 shRNA
7.5M Th17-Control shRNA
7.5M Th17-Entpd1 shRNA
K
B16 10%TRP-1-KO tumor model
Survival rate (%)
Days after tumor inoculation
20 M Th1-Control shRNA
20 M Th1-Entpd1 shRNA
7.5 M Th17-Control shRNA
7.5 M Th17-Entpd1 shRNA
L
Relative Tyrp1 expression
B16 tumor
B16 10%TRP-1-KO tumor
20M Th1-Control shRNA
7.5M Th17-Entpd1 shRNA
7.5M Th17-Control shRNA
B16 10%TRP-1-KO tumor
M
B16 40%TRP-1-KO tumor model
Survival rate (%)
Days after tumor inoculation
Only CTX
Th9+Vehicle
Th9+POM-1

Figure S4, related to Figure 4. Purinergic receptor P2Y2 signaling is crucial for the anti-ALV response of Th9 ACT.

(**A-C**) TRP-1-specific Th9 cells (2.5×10^6) were transferred i.v. into WT mice or *P2ry2*$^{-/-}$ mice bearing 10-day established B16$^{10\%TRP\text{-}1\text{-}KO}$ tumors (1×10^6 B16$^{10\%TRP\text{-}1\text{-}KO}$ cells challenged s.c. 10 days before T cell transfer). Adjuvant cyclophosphamide (CTX, i.p.) and DC vaccination (2.5×10^5, i.v.) were administered to mice as indicated in Fig. 1A. (**A**) Survival curves are shown (n=9-10/group, combined from 2 independent experiments). $^{**}P<0.01$, compared with any other groups, survival analysis was conducted by log-rank test. Tumors were harvested at the endpoint and analyzed by qPCR for *Tyrp1* (**B**) (endpoint), *Ifna*, and *Ifnb* (**C**) (10 days after ACT) mRNA levels (n=3). Data are mean ± SD. $^{**}P<0.01$, compared with any other groups, one-way ANOVA with Tukey test.

(**D**-**E**) Monocytes were isolated from spleens of WT (CD45.1) or *P2ry2*$^{-/-}$ (CD45.2) tumor-free mice 10 days after CTX treatment. *P2ry2*$^{-/-}$ (CD45.2) mice bearing 10-day established B16$^{10\%TRP\text{-}1\text{-}KO}$ tumors (1×10^6 B16$^{10\%TRP\text{-}1\text{-}KO}$ cells challenged s.c.) were treated with CTX on day 9, followed by Th9 cell and DC transfer (similar to Fig. 1F). Monocytes (1×10^7) were transferred i.v. into mice on day 12 and day 22. (**D**) The number of recruited monocytes in tumors on day 20 after tumor challenge (n=3/group). Data are mean ± SD. $^{**}P<0.01$, compared with any other groups, one-way ANOVA with Tukey test. Tumor monocytes in WT monocyte-reconstituted mice after Th9 cell ACT were predominately CD45.1$^+$ monocytes. (**E**) Mice survival curves are shown (n=9-10/group, combined from 2 independent experiments). Survival analysis was conducted by log-rank test.

(**F**) Human Th9 cells were analyzed for intracellular staining of hIL-9.

(**G**) Human T cells were analyzed for surface expression of hCD39.

(**H**) The percentage of CD39 positive cells were analyzed by FACS (n=3). Data are mean ± SD. $^{**}P<0.01$, compared with CTRLshRNA groups, Student's *t*-test.

(**I-L**) TRP-1-specific T cells were transferred i.v. B6 mice bearing 10-day established B16$^{10\%TRP\text{-}1\text{-}KO}$ tumors (1×10^6 B16$^{10\%TRP\text{-}1\text{-}KO}$ cells challenged s.c. 10 days before T cell transfer). Adjuvant cyclophosphamide (CTX, i.p.) and DC vaccination (2.5×10^5, i.v.) were administered to mice as indicated in Fig. 1A. (**I**) The cell number of monocytes in the tumor was determined by FACS 10 days after ACT (n=4). Data are mean ± SD. $^{**}P<0.01$, compared with Th1 and Th17 Control shRNA groups, one-way ANOVA with Tukey test. (**J**) ELISpot analysis measuring IFNγ^+ spots derived from CD4$^+$ T cells, CD8$^+$ T cells, or NK cells isolated from ~200 mg tumors treated as indicated 20 days after ACT. The graph shows the average number of IFNγ^+ spots from triplicate wells (n=3). Data are mean ± SD. $^{**}P<0.01$, compared with Th1 Control shRNA group, one-way ANOVA with Tukey test. (**K**) Survival curves are shown (n=10/group, combined from 2 independent experiments). $^{*}P<0.05$, compared with any other groups, survival analysis was conducted by log-rank test. (**L**) Tumors at the endpoint were harvested and analyzed by qPCR for *Tyrp1* mRNA levels (n=3). Data are mean ± SD. $^{**}P<0.01$, compared with B16 tumor and B16$^{10\%TRP\text{-}1\text{-}KO}$ tumor groups, one-way ANOVA with Tukey test.

(**M**) TRP-1-specific Th9 cells were transferred i.v. to B6 mice bearing 10-day established B16$^{40\%TRP\text{-}1\text{-}KO}$ tumors (1×10^6 B16$^{40\%TRP\text{-}1\text{-}KO}$ cells challenged s.c. 10 days before T cell transfer). Adjuvant cyclophosphamide (CTX, i.p.) and DC vaccination (2.5×10^5, i.v.) were administered to mice as indicated in Fig. 1A. Vehicle control or CD39 inhibitor POM-1 (125 μg/mouse) was i.p injected every 3

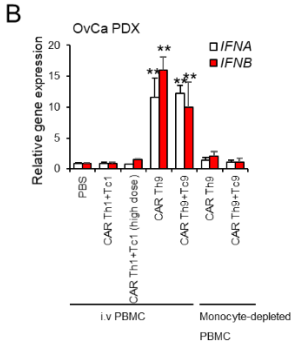
days starting from 5 days after ACT for a total of 15 doses. Survival curves are shown (n=9-12/group, combined from 2 independent experiments). Survival analysis was conducted by log-rank test.

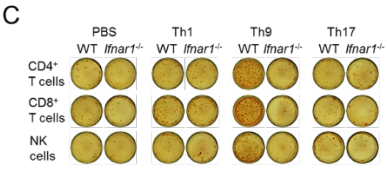
Representative results from one of two repeated experiments are shown.

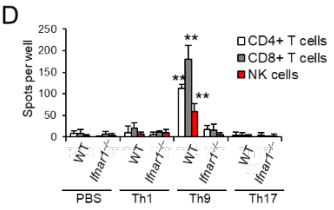
A

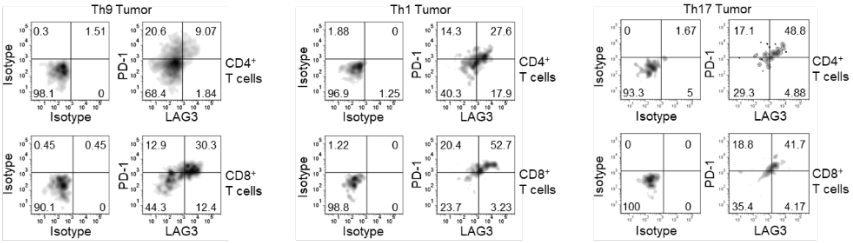
The top 10 most altered gene sets:

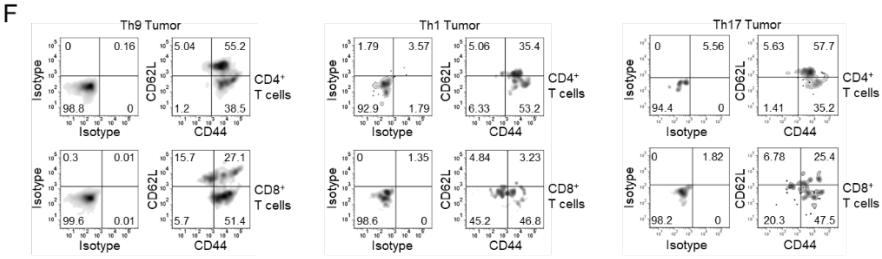
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION
KEGG_ANTIGEN_PROCESSING_AND_PRESENTATION
KEGG_JAK_STAT_SIGNALING_PATHWAY
KEGG_CHEMOKINE_SIGNALING_PATHWAY
KEGG_VIRAL_MYOCARDITIS
KEGG_REGULATION_OF_AUTOPHAGY
KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY
KEGG_MAPK_SIGNALING_PATHWAY
KEGG_ADIPOCYTOKINE_SIGNALING_PATHWAY
KEGG_PRIMARY_IMMUNODEFICIENCY

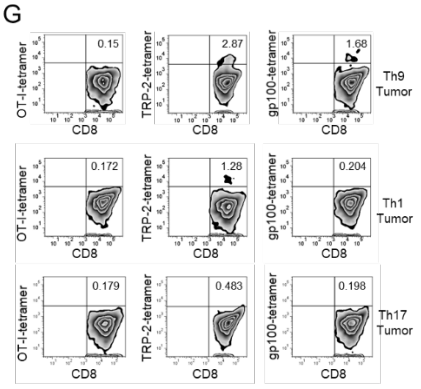


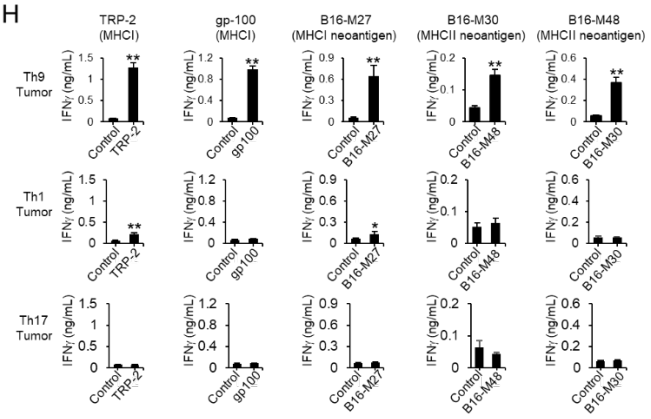


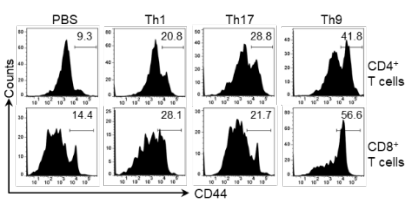


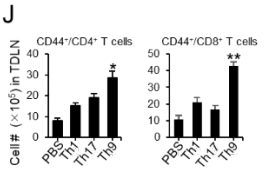


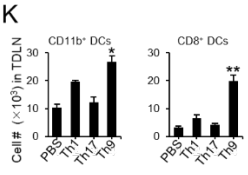


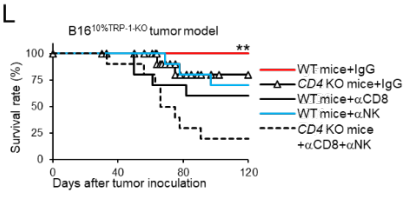












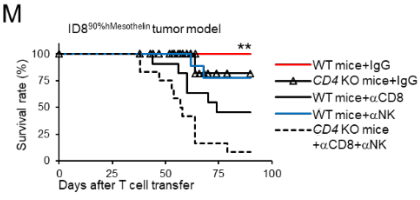


Figure S5, related to Figure 5. Th9 ACT induced activation of host T cell response.

(**A**) The top 10 most altered gene sets (Fig. 5A) of tumor monocytes compared to spleen monocytes from Th9 cell-treated group.

(**B**) OvCa PDX treated as in Fig. S2 were harvested from NSG mice 10 days after PBMC transfer. Some groups of mice received monocyte-depleted PBMC (hCD14-beads depletion) as indicated. Tumor tissues were analyzed by qPCR for relative gene levels (n=3). Data are mean ± SD. $^{**}P<0.01$, compared with PBS, Th1+Tc1, and monocyte-depleted PBMC groups, one-way ANOVA with Tukey test.

(**C**-**D**) ELISpot analysis measuring IFNγ^+ spots derived from CD4$^+$ T cells, CD8$^+$ T cells, or NK cells isolated from ~200 mg tumors treated as indicated 20 days after ACT. Representative data (**C**) and summarized results (n=4) (**D**) are shown. Data are mean ± SD. $^{**}P<0.01$, compared with any other groups, one-way ANOVA with Tukey test.

(**E**-**K**) TRP-1-specific Th cells were transferred i.v. to B6 mice bearing 10-day established B16$^{10\%\text{TRP-1-KO}}$ tumors (1×10^6 B16$^{10\%\text{TRP-1-KO}}$ cells challenged s.c. 10 days before T cell transfer). Adjuvant cyclophosphamide (CTX, i.p.) and DC vaccination (2.5×10^5, i.v.) were administered to mice as indicated in Fig. 1A. (**E**) Surface expression of indicated exhaustion markers on host T cells in the B16$^{10\%\text{TRP-1-KO}}$ tumors 20 days after Th9, Th1, or Th17 cell ACT was determined by FACS. Representative data are shown. (**F**) FACS analysis of CD62L and CD44 surface expression on host T cells in B16$^{10\%\text{TRP-1-KO}}$ tumors 20 days after Th9, Th1, or Th17 cell ACT. Representative data are shown. (**G**) Percentage of indicated tetramer$^+$ host CD8$^+$ T cells in B16$^{10\%\text{TRP-1-KO}}$ tumors 20 days after Th9, Th1, or Th17 cell ACT was determined by FACS. T cells were restimulated with peptide-loaded DCs for 72 hrs in the presence of IL-2 before staining. Representative data are shown. (**H**) CD4$^+$ T cells or CD8$^+$ T cells isolated from ~200 mg tumors were cocultured with peptide-loaded DCs for 72 hrs. The level of IFNγ was measured by ELISA in the supernatants (n = 3/group). Data are mean ± SD. $^{**}P<0.01$, compared to control group, Student's *t*-tests. (**I**) The percentage of CD44^{hi} T cells in the TDLN of B16$^{10\%\text{TRP-1-KO}}$ tumor-bearing mice 20 days after Th1, Th17, and Th9 cell ACT was determined by FACS. Representative (**I**) and summarized (**J**) data are shown. (**K**) CD11b$^+$ DCs (CD11b$^+$CD11c$^+$MHC-II^{hi}) and CD8$^+$ DCs (CD8$^+$CD11c$^+$MHC-II^{hi}) in the TDLN of B16$^{10\%\text{TRP-1-KO}}$ tumor-bearing mice 20 days after Th9 cell ACT were determined by FACS. The calculated total # of cells per TDLN is shown (n = 3/group). Data are mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, compared with any other groups, one-way ANOVA with Tukey test.

(**L**) TRP-1-specific Th9 cells were transferred i.v. into WT B6 or CD4-KO B6 mice bearing 10-day established B16$^{10\%\text{TRP-1-KO}}$ tumors (1×10^6 B16$^{10\%\text{TRP-1-KO}}$ cells challenged s.c. 10 days before T cell transfer). Adjuvant cyclophosphamide (CTX, i.p.) and DC vaccination (2.5×10^5, i.v.) were administered to mice as indicated in Fig. 1A. Control IgG, depletion antibody for CD8$^+$ T cells or NK cells were i.p. injected every 3 days until the endpoint starting on one day before ACT. Survival curves are shown (n=10/group, combined from 2 independent experiments). $^{**}P<0.01$, compared with CD4-KO mice+αCD8+αNK group, survival analysis was conducted by log-rank test.

(**M**) hMesothelin-mCAR murine Th9 cells (5.0×10^6 GFP$^+$) were transferred i.v. into WT B6 or *CD4* KO B6 mice bearing 45-day-established, s.c. injected ID8$^{90\%\text{hMesothelin}}$ tumors (containing 10% WT ID8 cells as ALVs). Adjuvant cyclophosphamide (CTX, i.p.) was administered to mice that received T cells. Control IgG, depletion antibody for CD8$^+$ T cells or NK cells were i.p. injected every 3 days until the endpoint starting on one day before ACT. Survival curves are shown (n=9-10/group, combined from 2

independent experiments). $^{**}P<0.01$, compared with *CD4* KO mice+αCD8+αNK group, survival analysis was conducted by log-rank test.

Representative results from one of two repeated experiments are shown.

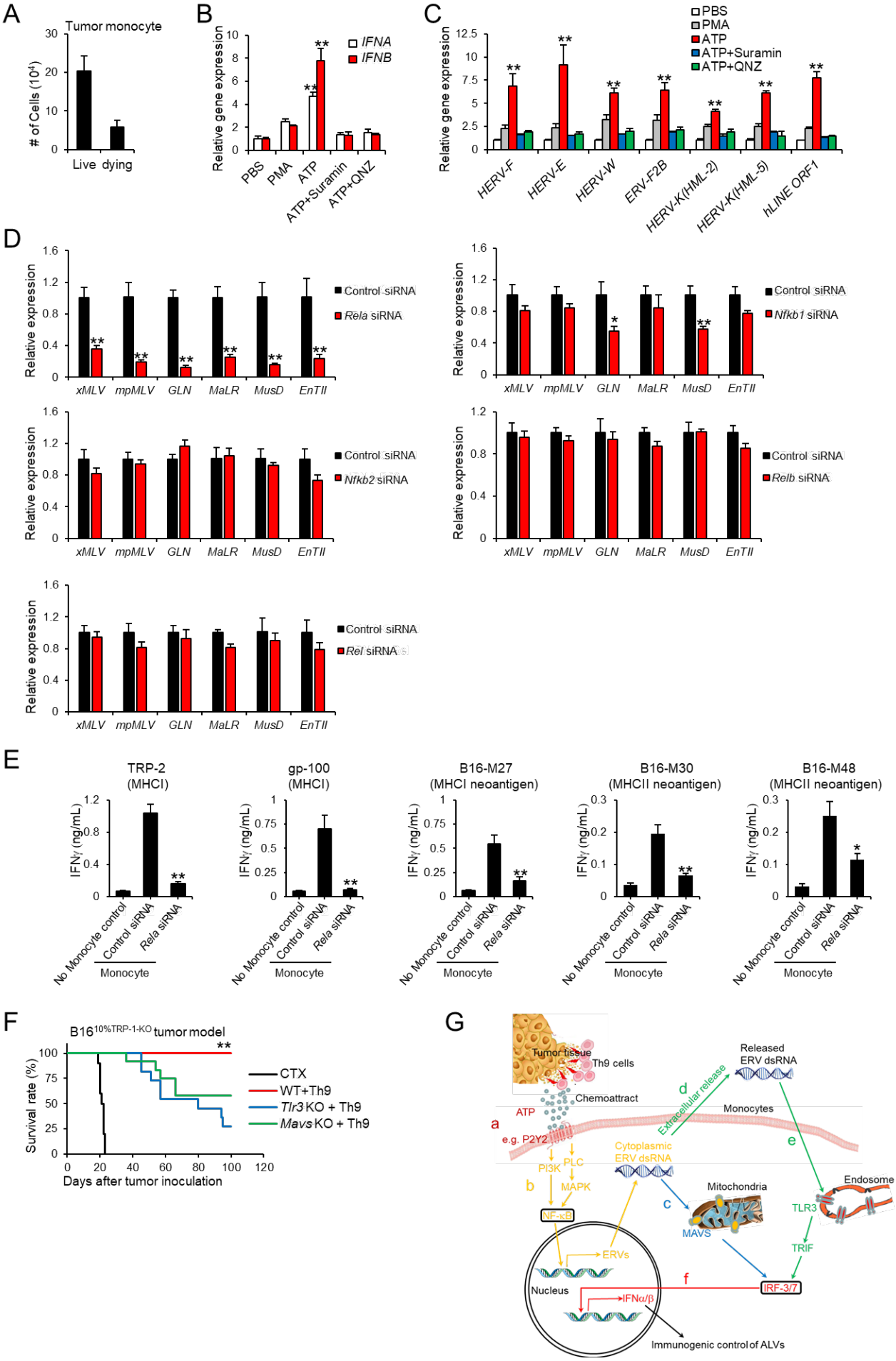

A
Tumor monocyte
# of Cells (10^4)
Live
dying
B
Relative gene expression
IFNA
IFNB
PBS
PMA
ATP
ATP+Suramin
ATP+QNZ
C
PBS
PMA
ATP
ATP+Suramin
ATP+QNZ
Relative gene expression
HERV-F
HERV-E
HERV-W
ERV-F2B
HERV-K(HML-2)
HERV-K(HML-5)
hLINE ORF1
D
Relative expression
Control siRNA
Rela siRNA
Nfkb1 siRNA
Nfkb2 siRNA
Relb siRNA
Rel siRNA
xMLV
mpMLV
GLN
MaLR
MusD
EnTII
E
TRP-2 (MHCI)
gp-100 (MHCI)
B16-M27 (MHCI neoantigen)
B16-M30 (MHCII neoantigen)
B16-M48 (MHCII neoantigen)
IFNγ (ng/mL)
No Monocyte control
Control siRNA
Rela siRNA
Monocyte
F
B16 10%TRP-1-KO tumor model
Survival rate (%)
Days after tumor inoculation
CTX
WT+Th9
Tlr3 KO + Th9
Mavs KO + Th9
G
Tumor tissue
Th9 cells
Chemoattract
ATP
e.g. P2Y2
a
PI3K
PLC
b
MAPK
NF-κB
ERVs
Nucleus
IFNα/β
Immunogenic control of ALVs
Cytoplasmic ERV dsRNA
c
Mitochondria
MAVS
d
Extracellular release
Released ERV dsRNA
Monocytes
e
Endosome
TLR3
TRIF
f
IRF-3/7

Figure S6, related to Figure 6. ERV induction in monocytes is required for anti-ALV immune response in Th9 cell-treated mice.

(**A**) B16 tumor-bearing mice were treated with TRP-1 Th9 cells as shown in Fig. 1F (main figure). Tumor tissues (200 mg/mice, 10 days after ACT) were harvested for FACS analysis. The total number of live (viability dye staining negative) and dying (viability dye staining positive) tumor monocytes (n=3-4/group) are shown.

(**B**-**C**) Human monocytic THP-1 cells were treated as indicated *in vitro*. Relative gene expression was determined by qPCR (n=4). Data are mean ± SD. $^{**}P<0.01$, compared with any other groups, one-way ANOVA with Tukey test.

(**D**-**E**) Monocytes were isolated from spleens of WT tumor-free mice 10 days after CTX treatment. *P2ry2*$^{-/-}$ mice bearing 10-day established B16$^{10\%\text{TRP-1-KO}}$ tumors (1×10^6 B16$^{10\%\text{TRP-1-KO}}$ cells challenged s.c.) were treated with CTX on day 9, followed by Th9 cell and DC transfer (similar to Fig. 1F). Monocytes (1×10^6) were transduced with siRNA and intratumorally injected into tumors 7 and/or 14 days after Th9 ACT. (**D**) Intratumorally injected WT monocytes were sorted from tumors on day 10 after Th9 cell transfer for testing transcript levels of the indicated murine ERVs measured by RT-qPCR (n=3). Data are mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, compared with Control siRNA group, Student's *t*-tests. (**E**) *Rela* targeting siRNA transduced monocytes were intratumorally injected 7 and 14 days after Th9 ACT. A group of mice without intratumoral injection of monocytes was included as a control (No Monocyte control). Host CD4$^+$ T cells and CD8$^+$ T cells isolated from ~200 mg tumors 20 days after ACT were cocultured with peptide-loaded DCs for 72 hrs in the presence of IL-2. The level of IFNγ was measured by ELISA in the supernatants (n = 3/group). Data are mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, compared with Control siRNA group, one-way ANOVA with Tukey test.

(**F**) TRP-1-specific Th9 cells were transferred i.v. into WT B6, *Tlr3* KO, or *Mavs* KO B6 mice bearing 10-day established B16$^{10\%\text{TRP-1-KO}}$ tumors (1×10^6 B16$^{10\%\text{TRP-1-KO}}$ cells challenged s.c. 10 days before T cell transfer). Adjuvant cyclophosphamide (CTX, i.p.) and DC vaccination (2.5×10^5, i.v.) were administered to mice as indicated in Fig. 1A. Survival curves are shown (n=10-12/group, combined from 3 independent experiments). $^{**}P<0.01$, compared with any other groups, survival analysis was conducted by log-rank test.

(**G**) The proposed mechanism of anti-ALV immunity induction by Th9 cell ACT. (**a**) Effector function of Th9 cell ACT results in releasing of extracellular ATP (eATP) from dying tumor cells. eATP will recruit monocytes into tumors through ATP-P2Y2 interaction. (**b**) Downstream signaling of ATP-purinergic receptor activates NF-κB signaling, which leads to transcriptional activation of numerous endogenous retrovirus (ERV) genes. (**c**) Cytoplasmic ERV dsRNAs activate the MAVS pathway. (**d**) ERV dsRNAs may be released into the extracellular milieu from activated or damaged tumor-infiltrating monocytes. (**e**) Released ERV dsRNAs activate the TLR3/TRIF pathway in tumor-infiltrating monocytes. (**f**) TLR3 and MAVS pathway activation stimulates type I IFN production in tumor-infiltrating monocytes, which mediate the immunogenic killing of ALVs.

Representative results from one of two repeated experiments are shown.