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Supplementary Information for

Induction of oligoclonal CD8 T cell responses against pulmonary metastatic cancer by a phospholipid-conjugated TLR7 agonist

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Supplementary Information Text

SI Appendix Figure Legends

Fig. S1

Systemic administration of 1V270 inhibits lung metastasis, but not the primary tumor growth

(A) Growth curves of the orthotopically implanted primary tumor in the spontaneous metastasis model.4T1 cells (5×10^5) were inoculated to both 4^{th} mammary pads of BALB/c mice (n=13/group). 1V270 (20, 80, or 200 μg/injection) was i.p. administered on days 7, 10, 14, 17, 21, and 24 as shown in Figure 1A. Tumor growth was measured with a caliper and calculated using the formula: volume (mm^3)=$(width)^2 \times length/2$. (B) Experimental protocol of T cell depletion. BALB/c mice (n=6-15/group) were i.p. treated with anti-CD8-mAb. (C) Growth curves of the orthotopically implanted primary tumor in the $CD8^+$ T cells depleted mice. Data shown are mean ± SEM and representative of two independent experiments showing similar data.

Fig. S2

Representative gating process of $CD8^+$ T cells in TILs by flow cytometry

(A) Representative gating process of $CD8^+$ T cells ($CD45^+CD3^+CD8^+$). (B) Representative flow cytometric plots of $CD8^+$ T cells on day 26 following $CD8^+$ T cell depletion. Over 80 % $CD8^+$ T cell population was depleted both in vehicle and 1V270 treated groups.

Fig. S3

1V270 therapy increases frequency of commonly shared clones between individuals

(A) The clonality index (1-normalized Shannon index) of $CD8^+$ T cells infiltrating to left side of the secondarily challenged tumor were plotted against both sides of tumor volume in the no-

tumor exposed mice. The correlation was evaluated by a Pearson's correlation test (R^2=0.15, p=0.61). (B) The Venn diagram of "shared clones" among the individual mice in the same group. Based on the sequence of CDR3 region, number of TCR clones shared between individual mice were counted. The shared clone was defined as the TCR clone consisting of identical V and D genes and amino acid sequence of CDR3 shared among 3 or more mice in the groups. (C) The percentage frequency of shared clones in the total reads in TILs. The frequency was calculated by dividing the sum of number of sequence reads from shared clones by the total number of reads. Each dot represents the frequency of the shared clones of either TCRα (green) or TCRβ (blue) in the individual mice.

Fig. S4

Intraperitoneal administration of 1V270 activates local dendritic cells in the lungs

(A) Representative gating process of $CD11c^+$MHC $classII^+$ dendritic cells in the lungs. (B) BALB/c mice (n=5/group) were treated with 1V270 (200 μg/injection) on day -1 and then tumor cells were i.v. administered on day 0. Seven days later, single cell suspensions derived from the lungs were stained for $CD11c^+$MHC class II^+ dendritic cells (left panel). The cells were further assayed for costimulatory molecules (CD80, and CD86) expression (right panel). Each dot indicates an individual mouse and horizontal bars indicate means. *P<0.05 by Kruskal-Wallis test with Dunn's *post hoc* test comparing treatment groups against vehicle.

Fig. S5

In vivo imaging in the IV metastasis model

(A) In vivo imaging protocol in IV metastasis model. (B) Representative of lung tumor signals by IVIS in Figure4D.

Fig. S6

1V270 therapy recruits innate immune cells to the lungs

(A-C) Representative flow cytometric plots and gating process of immune cells in the lungs. BALB/c mice (n=6-7/group) were treated with 1V270 (200 μg/injection) on day -1 and then tumor cells were i.v. administered on day 0. On day 7, single cell suspensions derived from the lungs were stained for (A) NK cells ($CD45^+CD3^-NKp46^+CD49^+$), (B) M-MDSCs ($CD45^+CD11b^+Ly6G^-Ly6C^{high}$) and (C) PMN-MDSCs ($CD45^+CD11b^+Ly6G^+$) were analyzed by flow cytometric assay. (C) Frequencies of M-MDSCs and PMN-MDSCs were compared between 1V270 treated and vehicle-treated mice. Each dot indicates an individual mouse and horizontal and vertical bars indicate means ± SEM. Data are representative of 2 independent experiments showing similar results. Mann-Whitney U test was used to compare treatment groups against the vehicle group. *P<0.05, **P<0.01.

Fig. S7

Antibody-mediated depletion of NK cells reverses inhibition of tumor cell colonization by 1V270 therapy in the early phase

(A) Experimental protocol of NK cell depletion. Anti-asialo GM1 rabbit polyclonal Ab (aGM1) or rabbit IgG polyclonal Ab was injected on days -4, -1, 3, and 10. 1V270 (200 μg/injection) was administered on day -1 and 2×10^4 4T1 cells were i.v. injected on day 0. (B) Representative flow cytometric plots of NK cell frequency in the lung on day 14. (C) Representative of lung tumor signals of NK cell depleted mice by IVIS in Figure 4F.

Fig. S8

Intraperitoneal 1V270 treatment activates $CD11c^+$ DCs in medastinal LN but not DCs in cervical and inguinal LNs

1V270 (200 µg/injection) was administered one day prior to i.v. administration of 4T1 cells (2×10^4). Mice were euthanized on days 7, 14, and 22. (A) Total numbers of CD11c+ DC in the medicinal LNs were compared between 1V270 and vehicle treatments. *P=0.0004 by two-way ANOVA followed by Bonferroni's multiple comparison test. (B, C and D) Total (B), $CD80^+$ (C), or $CD86^+$ (D) $CD11c^+$ DC in the medicinal, cervical, and inguinal LNs on day 7 were compared between 1V270 and vehicle treatments. (E and F) Total numbers of $CD8^+$ T cells (E) and $PD\text{-}1^+$ $CD8^+$ T cells (F) in the medicinal, cervical, and inguinal LNs on day 7 were compared between 1V270 and vehicle treatments were plotted. *P<0.05 by Mann-Whitney U test. Data shown are representative of two independent experiments showing similar trends.

Fig. S9

Minimal body weight loss induced by administration of therapeutic dose of 1V270.

A) C57BL/6 mice were i.v. or i.p. injected with 71 mg/kg (MTD) 1V270 and body weight was monitored. B) BALB/c mice were administered 20 and 200 nmol/25g mouse (corresponding to 0.8, and 8 mg/kg, respectively) 1V270 and body weight was measured next day. C) $2 \times 10^4$4T1 cells were i.v. injected on day 0 and 1V270 therapy (8, 0.8, and 0.08 mg/kg, corresponding to 200, 20, and 2 nmol/25g, respectively) was administered on days -1, 7, 10, 14, 17 and 21. None of these doses significantly caused weight loss. D) Wild type C57BL/6 mice or *Tlr7*$^{-/-}$ mice were i.v. injected with 71 mg/kg 1V270 and body weight was monitored. *P<0.05 by two-way ANOVA and Dunn's post hoc test.

Fig. S10

Toll-like receptor 7 expression in 4T1, B16, LLC and 3T3 cells

Expression of TLR7 in the cell lines used in this study was assessed by quantitative RT-PCR.

Fig. S11

Systemic administration of 1V270 induces significantly lower levels of cytokine induction by systemically administrated 1V270 and 1V136

BALB/c mice (n=5) were i.p. administered 1V270 (200μg=185 nmol/injection) or 1V136 (58 μg =185 nmol/injection) and sera were collected 2, 4, 6, and 24 h following the administration. The levels of TNFα, IL-12, and IP-10 were measured by Luminex assay. *P<0.05, **P<0.01 by two-way ANOVA using a Bonferroni *post hoc* test comparing treatment groups.

Fig. S12

The correlation between the tumor signal in the lung at day 10, and the number of lung nodules or overall survival

(A) BALB/c mice (n=5) were injected with 2×10^4 4T1 cells on day 0. Tumor cell signals were monitored using IVIS. Tumor signals on day 10 were plotted with the number of lung tumor nodules examined on day 21. (B) BALB/c mice (n=8 /group) were injected with 2×10^4 4T1 cells on day 0. Tumor signals were monitored using IVIS. Tumor signals on day 10 were plotted with survival (days) of individual mice. The representative tumor signal images by IVIS for (A) and (B) are shown at right. The correlation was evaluated by a Pearson's correlation test.

SI Appendix, Method

4T1 breast tumor models

5×10^5 4T1 cells were inoculated in both sides of the 4th mammary pads of female BALB/c mice purchased from Charles River Laboratories (Wilmingtin, MA) . Tumor length and width were recorded, and tumor volumes were calculated using the formula: volume (mm^3)=$(width)^2\times length$ /2. On day 28, mice were euthanized and the lungs were stained with intratracheally injected India ink and destained in Fekete's solution to count tumor nodules. In the experimental metastasis model, 2×10^4 4T1 cells were i.v. injected. On day 21, mice were euthanized and tumor nodules in lungs were counted as described above. In the secondary challenge experiment, 1V270 treated- mice without tumor signals in the lungs on day 21 of the experimental metastasis protocol were included. 5×10^5 4T1 cells were inoculated in both sides of the 4th mammary pads. Tumor length and width were recorded, and tumor volumes were calculated similar with spontaneous metastasis model.

Histologic analysis

Lungs were fixed with 10% formalin for overnight, dehydrated and embedded in paraffin on the Excelsior ES tissue processor (Thermo Scientific) and sectioned at 5 um thickness on a rotary microtome. Antigen retrieval was performed in Citrate buffer, pH 6.0, heated to 98C for 8 minutes and cooled at room temperature for 20 minutes. The sections were stained on a Lab Vision 360 automated immunostaining instrument (Thermo Scientific) using a 2 step immuno-peroxidase protocol. Briefly, the slides were blocked for endogenous peroxidase activity, washed and incubated with protein blocking buffer, 5% normal donkey serum in TBS for 10 minutes. The primary antibodies, both rabbit polyclonal, CD45 (ab10558, AbCam, used at 1 ug/ml diluted in 5% NDS) and CD3 (ab16669 from AbCam at a 1:100 dilution) were incubated for 1 hour at room temperature. After washing in TBS-tween, the slides were incubated with the secondary antibody - HRP-Donkey F(ab')2 anti rabbit (Jackson ImmunoResearch Laboratories diluted 1:200) for 30 minutes at room temperature, washed and reacted with DAB as the brown color

substrate, hematoxylin was used the counterstain. Antibody details are shown in Supplemental Table 2. Images were acquired using Axio Imager Zeiss microscope (Zeiss, Thornwood, NY).

Flow cytometric analysis

To make the single cell suspensions, tumors were dissociated using the mouse tumor dissociation kit (Miltenyi Biotec, San Diego, CA) and the gentle MACS Octo Dissociator according to the manufacture's protocol. Single cell suspensions of spleens, lungs and mLN were prepared in Hank's Balanced Salt Solution (HBSS) supplemented with 20 μg/mL DNase I (Worthington, Lakewood, NJ) and 0.6 mg/mL collagenase type I (Worthington). Total cell number was counted by the ViaCount assay (MilliporeSigma, Darmstadt, Germany). Dead cells were excluded by propidium iodide staining.

NK cells or $CD8^+$ cell depletion *in vivo*

For NK cell depletion, 50 μL of anti-asialo GM1 rabbit polyclonal antibody (Wako, Richmond, VA) or rabbit IgG polyclonal antibody (Millipore, Temecula, CA) was injected on days -1, 1, 5, 9, 13, and 17. Mouse anti-CD8 (clone2.43) and isotype control Ab (clone LFA-2) were purchased from BioXcell (West Lebanon, NH). Anti-CD8 and isotype control (200μg/dose) were i.p administered on days 5, 8, 11, 14,16, 19, and 23 to mice.

Safety assessment of 1V270 *in vivo*

Wild type C57BL/6 and BALB/c were purchased from Charles Rive Laboratories. Tlr7 deficient mice (C57BL/6 background) were kindly gifted by Dr Shizuo Akira and bred and maintained by the UCSD Animal Care Program. In the single dose toxicity study, wild type Tlr7 deficient C57BL/6 (males and females) were i.p. injected with MTD (71 mg/kg), or effective doses (0.8 or 8 mg/kg) 1V270 on day 0 and body weight was monitored. In the repeated dose safety study, BALB/c mice were injected with 2×10^4 4T1 cells on day 0 and 1V270 therapy (8, 0.8, and 0.08 mg/kg, corresponding 200, 20, and 2 nmol/25g, respectively) was administered on days -1, 7, 10, 14, 17 and 21 and body weight was monitored. For complete blood count (CBC) and serum chemistry study, BALB/c mice (female 7-8 week old) were i.p. injected with 20, or

200nmol/injection 1V270 and blood was collected 18 h later using EDTA coated collection tubes. CBC and serum chemistry studies were performed by STAT Veterinary Laboratory (San Diego CA). Blood chemistry was assayed by ACP Diagnostic Veterinary Laboratory (UCSD). In the cytokine release study, the sera were collected from mice receiving 200 nmol of 1V136, 1V270 or vehicle 2, 4, 6, and 24 h after i.p. administration (1). Cytokine levels in sera were determined by Luminex bead assays (MILLIPLEX™ MAP kit, Millipore, Billerica, MA).

Assay for off-target binding of 1V270

Off target bindng assessment of 1V270 was performed by Eurofin Pharma Discovery Service (St Charles, MO).

Statistical analysis

Means and standard errors of means (SEM) are shown in other analyses. In dot plots, each dot represents a tumor, a spleen, or a lymph node from an individual mouse and the horizontal and vertical bars indicate mean and mean ± SEM. Mann-Whitney test was used to compare two groups. Using tumor volumes collected over all time points, two-way repeated measures ANOVA was used to compare different groups, with pair-wise contrasts made at the final time point using a Bonferroni *post hoc* test. To compare cross-sectional outcomes among more than two groups, Kruskal-Wallis tests with Dunn's *post hoc* test were applied. Correlations between tumor volumes and TCR repertoire analysis data were analyzed using a Pearson's correlation test, pooling data across the different treatment groups. Analysis of covariance (sometimes on the log scale) was used to test whether the correlation was mediated by differences among the treatment groups in both mean immune marker level and tumor volumes. $p < 0.05$ were considered statistically significant. Prism 6 (GraphPad Software, San Diego, CA) statistical software was used to carry out these analyses.

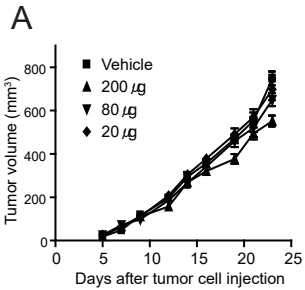
The laboratory operation and ethics statement

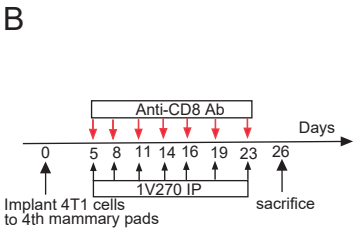
This study was performed using established laboratory protocols including tumor dissociation, processing, freezing, storage and thawing of cells as well as the staining procedure, data

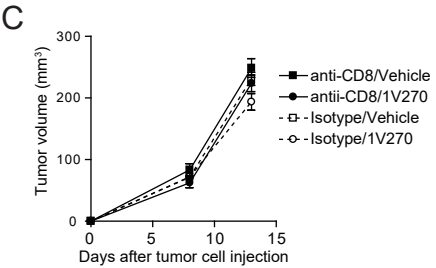
acquisition and gating strategy. The studies involving animal use were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of University of California, San Diego (PHS Animal Welfare Assurance Number: A3033-01; Protocol Numbers: S00028, and S05016). Mice were sacrificed by CO_2 inhalation followed by cervical dislocation. All efforts were made to minimize suffering during the procedures in this project.

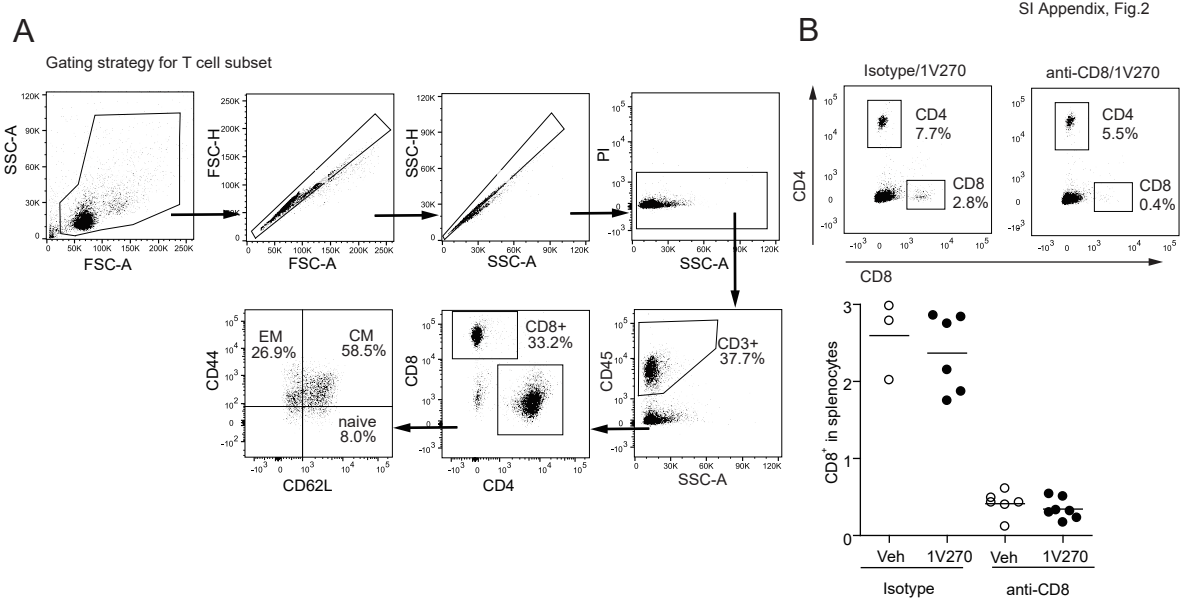
Reference

1. Chan M*, et al.* (2009) Synthesis and immunological characterization of toll-like receptor 7 agonistic conjugates. *Bioconj Chem* 20:1194-1200.

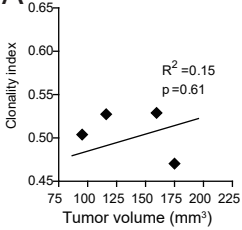

A
Vehicle
200 μg
80 μg
20 μg
Tumor volume (mm³)
800
600
400
200
0
0
5
10
15
20
25
Days after tumor cell injection


B
Anti-CD8 Ab
Days
0
5
8
11
14
16
19
23
26
1V270 IP
Implant 4T1 cells
to 4th mammary pads
sacrifice


C
Tumor volume (mm³)
300
200
100
0
0
5
10
15
Days after tumor cell injection
anti-CD8/Vehicle
antii-CD8/1V270
Isotype/Vehicle
Isotype/1V270

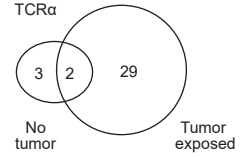

A
Gating strategy for T cell subset
SSC-A
FSC-A
FSC-H
FSC-A
SSC-H
SSC-A
PI
SSC-A
CD3+
37.7%
CD45
SSC-A
CD8+
33.2%
CD8
CD4
EM
26.9%
CM
58.5%
naive
8.0%
CD44
CD62L
B
Isotype/1V270
anti-CD8/1V270
CD4
7.7%
CD8
2.8%
CD4
5.5%
CD8
0.4%
CD4
CD8
CD8+ in splenocytes
Veh
1V270
Veh
1V270
Isotype
anti-CD8

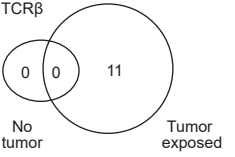
A



B

Number of TCR clones shared among 3 or more mice





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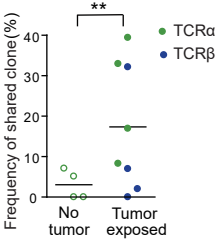
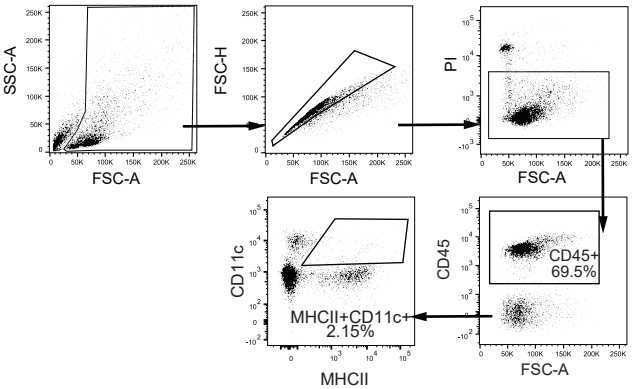
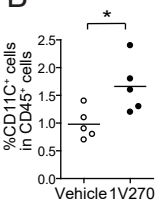


A Gating strategy for dendritic cells



7 day after tumor injection in lung FACS

B



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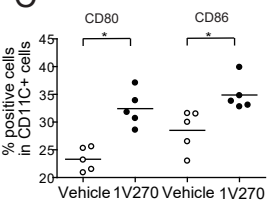
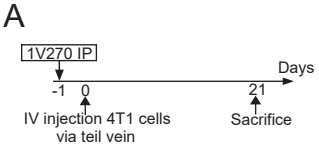
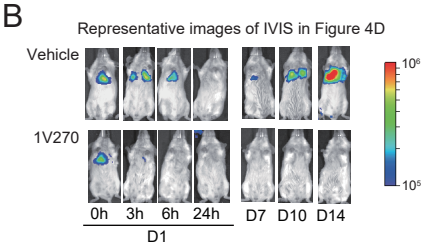
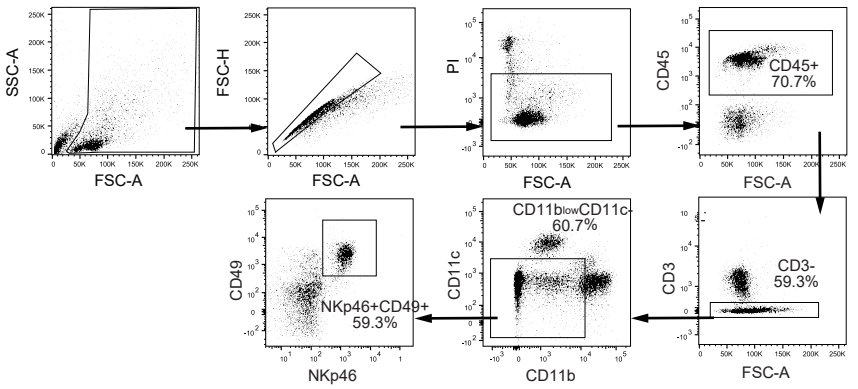


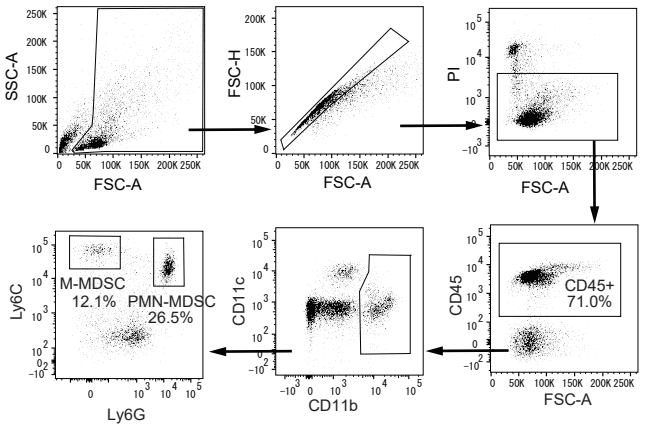

A
1V270 IP
Days
-1
0
21
IV injection 4T1 cells
via teil vein
Sacrifice


B
Representative images of IVIS in Figure 4D
Vehicle
1V270
10^6
10^5
0h
3h
6h
24h
D7
D10
D14
D1

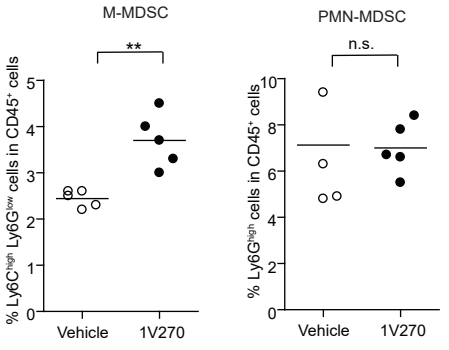
A Gating strategy for NK cells

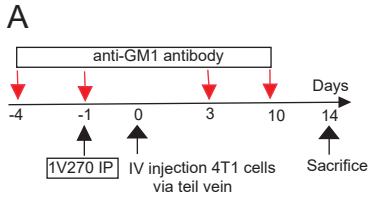


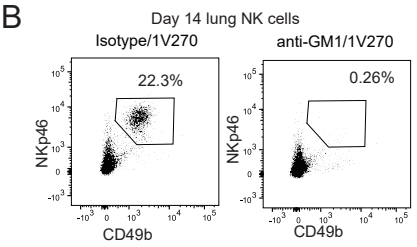
B Gating strategy for M-MDSCs and PMN-MDSCs

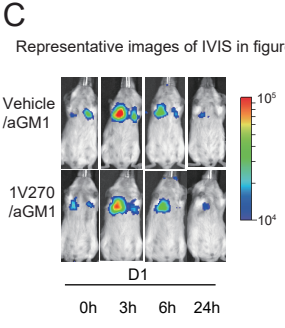


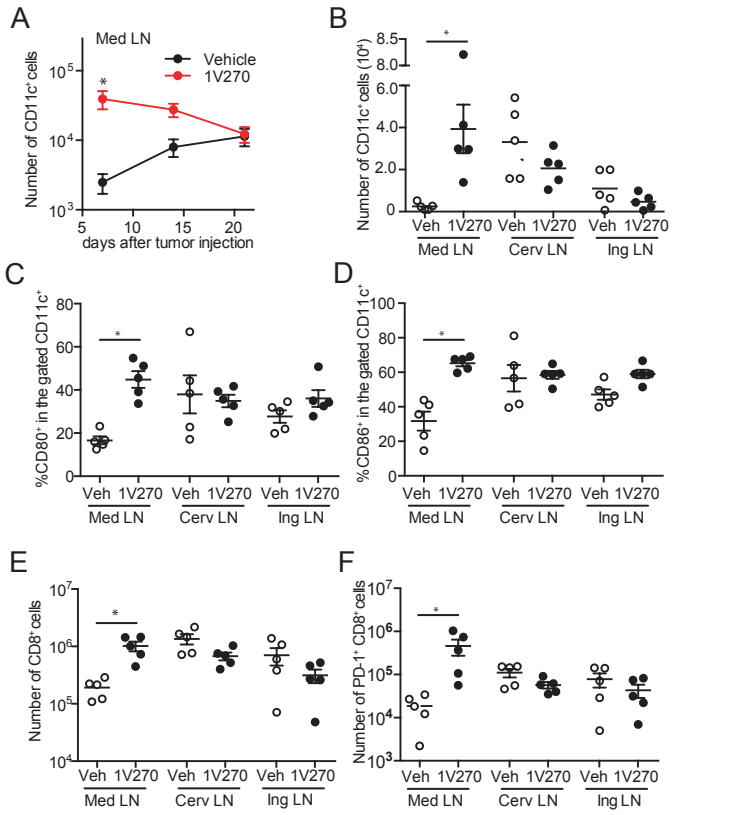
C 7 day after tumor injection in lung FACS

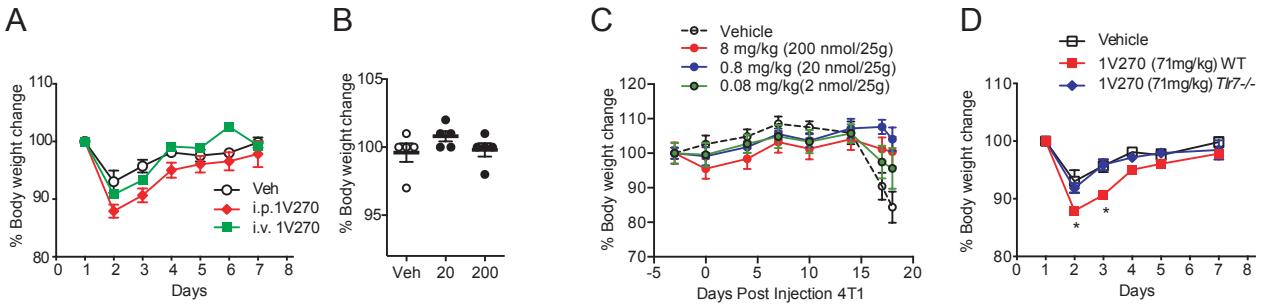



A
anti-GM1 antibody
Days
-4
-1
0
3
10
14
1V270 IP
IV injection 4T1 cells
via teil vein
Sacrifice

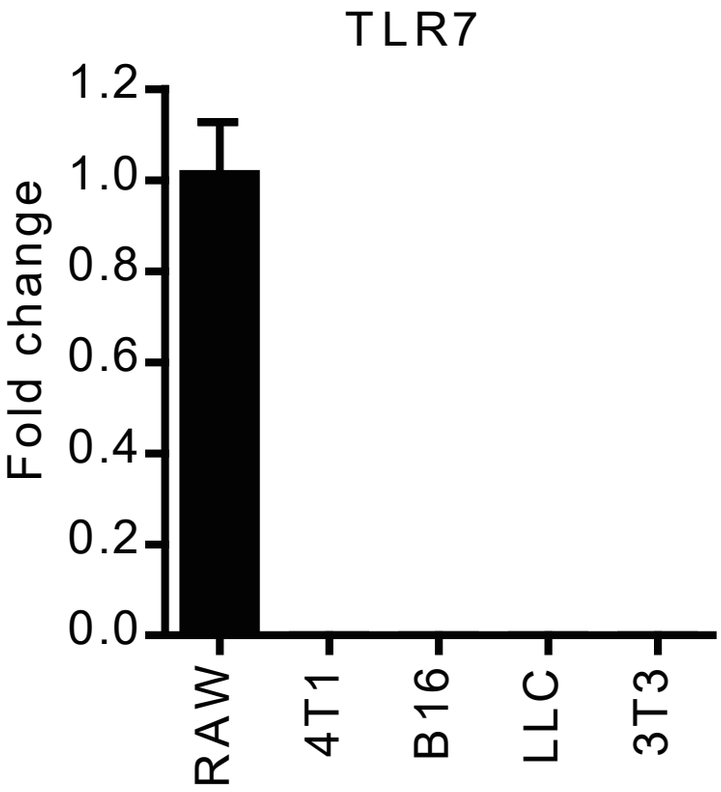

B
Day 14 lung NK cells
Isotype/1V270
anti-GM1/1V270
22.3%
0.26%
NKp46
CD49b

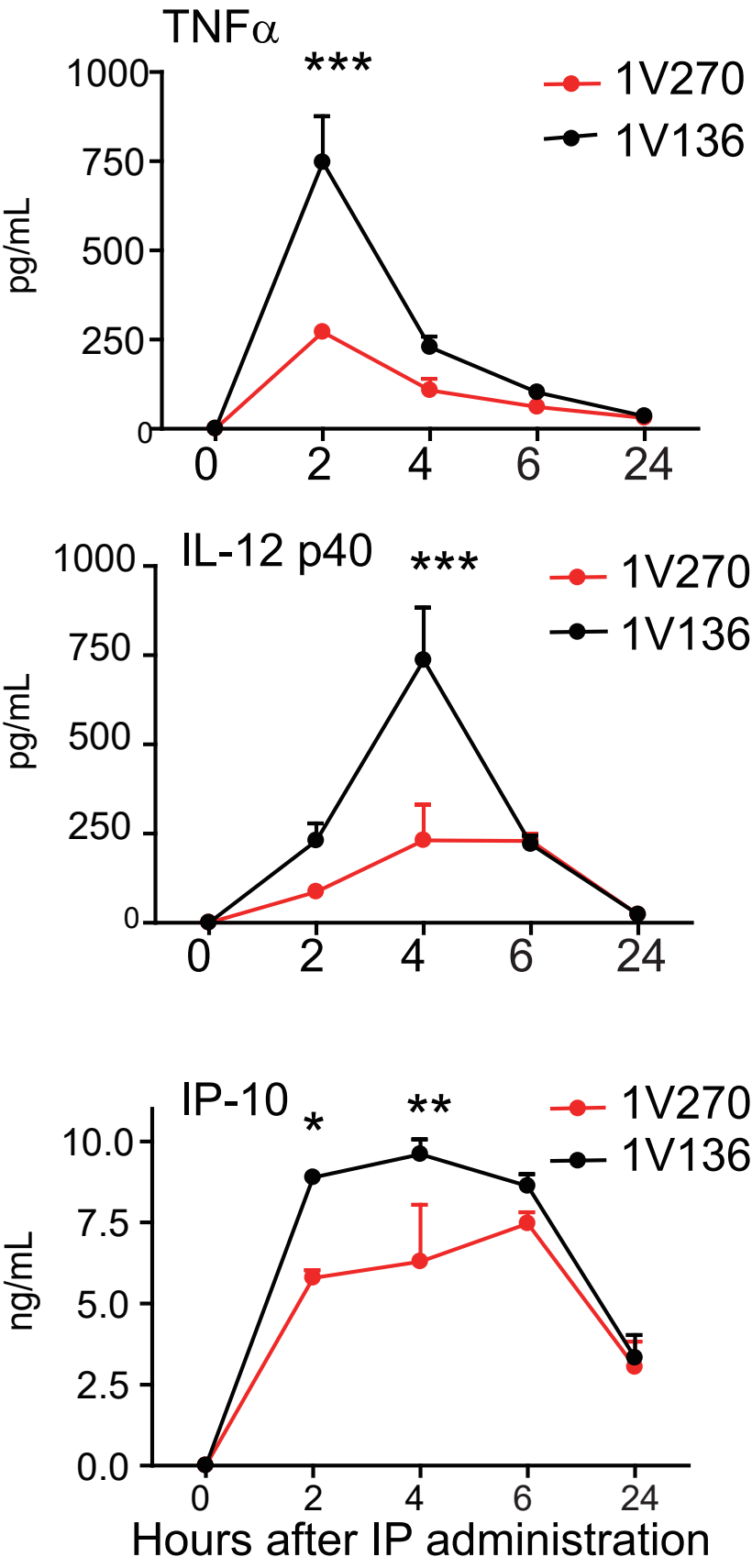

C
Representative images of IVIS in figure 4F
Vehicle
/aGM1
1V270
/aGM1
D1
0h
3h
6h
24h


A
Med LN
Vehicle
1V270
Number of CD11c+ cells
days after tumor injection
B
Number of CD11c+ cells (10⁶)
Veh 1V270 Veh 1V270 Veh 1V270
Med LN Cerv LN Ing LN
C
%CD80+ in the gated CD11c+
D
%CD86+ in the gated CD11c+
E
Number of CD8+ cells
F
Number of PD-1+ CD8+ cells

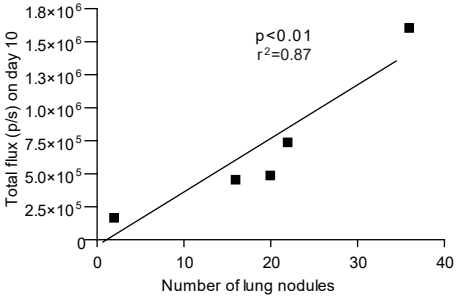

A
% Body weight change
Days
Veh
i.p.1V270
i.v. 1V270
B
% Body weight change
Veh
20
200
C
Vehicle
8 mg/kg (200 nmol/25g)
0.8 mg/kg (20 nmol/25g)
0.08 mg/kg(2 nmol/25g)
% Body weight change
Days Post Injection 4T1
D
Vehicle
1V270 (71mg/kg) WT
1V270 (71mg/kg) Tlr7-/-
% Body weight change
Days

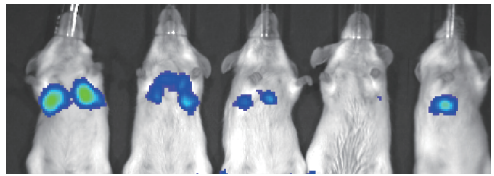
SI Appendix, Fig.10




TNFα
***
1V270
1V136
1000
750
500
250
0
pg/mL
0
2
4
6
24
IL-12 p40
***
1V270
1V136
1000
750
500
250
0
pg/mL
0
2
4
6
24
IP-10
*
**
1V270
1V136
10.0
7.5
5.0
2.5
0.0
ng/mL
0
2
4
6
24
Hours after IP administration

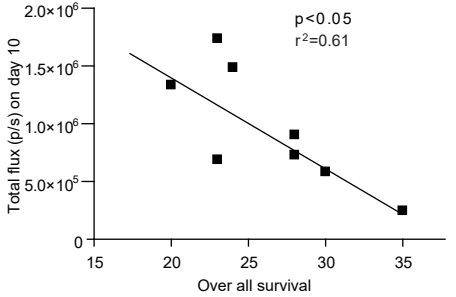
A

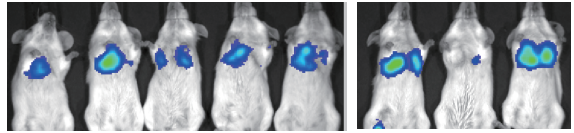




Total flux at day10	1.60E+06	7.34E+05	4.51E+05	1.62E+05	4.84E+05
Number of lung nodules	36	22	16	2	20

B





Total flux at day10	5.8.E+05	1.3.E+06	6.9.E+05	7.3.E+05	9.0.E+05	1.5.E+06	2.5.E+05	1.7.E+06
Overall survival	30	20	23	28	28	24	35	23

SI Appendix Table1 Antibodies used in flow cytometry analysis

Antibody	Color	Cat#	Clone	Host/Isotype	Vendor
CD3	PE/Cy7	552774	145-2c11	Armenian Hamster IgG1, κ	BD Biosciences
CD4	APC	17-0042	RM4-5	Rat IgG2a, κ	eBioscience
CD8a	e450	48-0081	53-6.7	Rat IgG2a, κ	eBioscience
CD11b	FITC	11-0112	M1/70	Rat IgG2b, κ	eBioscience
CD11b	e450	48-0112	M1/70	Rat IgG2b, κ	eBioscience
CD11c	APC/Cy7	117324	N418	Armenian Hamster IgG	Biolegend
CD44	APC/Cy7	103028	IM7	Rat IgG2b, κ	Biolegend
CD45	PE/Cy7	103114	30-F11	Rat IgG2b, κ	Biolegend
CD49b	PE	553858	DX5	Rat Lewis IgM, κ	BD Biosciences
CD62L	FITC	11-0621	MEL-14	Rat IgG2a, κ	eBioscience
CD80	FITC	104706	16-10A1	Armenian Hamste IgG	Biolegend
CD86	PE	12-0862	GL1	Rat IgG2a, κ	eBioscience
CD279(PD-1)	PE	135205	29F.1A12	Rat IgG2a, κ	Biolegend
CD335(NKP40)	FITC	560756	29A1.4	Rat IgG2a, κ	BD Biosciences
F4/80	FITC	11-4801	BM8	Rat IgG2a, κ	eBioscience
LY6C	Pacific blue	128014	HK1.4	Rat IgG2c,κ	Biolegend
LG6G	APC	127614	1A8	Rat IgG2a, κ	Biolegend
MHC Class2(I-ad)	APC	17-5323	AMS-32.1	Mouse IgG2b, k	eBioscience
Granzyme B	FITC	515403	GB11	Mouse IgG1, κ	BD Biosciences
IFNr	APC	17-7311	XMG1.2	Rat IgG1, κ	eBioscience

SI Appendix Table 2. Complete blood counts and blood chemistry

	Vehicle	1V270 20nmol	1V270 200nmol	Reference range	Unit
WBC	5.2 ± 0.4	5.5 ± 1.4	5.3 ± 0.3	5.4-16	$\times 10^3/\mu L$
RBC	10.7 ± 0.2	10.4 ± 0.1	9.7 ± 0.3*	6.7-12.5	$\times 10^6/\mu L$
HCT	51 ± 1	50 ± 1	47 ± 2	32-54	%
MCV	48 ± 0.3	48 ± 0.6	48 ± 0.7	31-62	fL
MCH	16 ± 0.2	16 ± 0.1	16 ± 0.1	n.s.	pg
MCHC	33.4 ± 0.5	33.6 ± 0.5	33.5 ± 0.6	22-35.5	g/dL
Platelet	668 ± 96	762 ± 46	769 ± 47	500-1500	$\times 10^3/\mu L$
Neutrophils	865 ± 136	1528 ± 392	1508 ± 147*	600-6100	/μL
% Neutrophils	17 ± 1	27 ± 2*	29 ± 2*	8-43	%
Lymphocytes	4062 ± 255	3735 ± 933*	3549 ± 299*	3300-6640	/μL
% Lymphocytes	79 ± 2	69 ± 2*	67 ± 3*	55-95	%
Monocytes	69 ± 9	80 ± 14	112 ± 25	0-1500	/μL
Alkaline phosphatase	186 ± 5	127 ± 9*	125 ± 8*	n.s.	unit/L
Alanine transaminase	46 ± 4	43 ± 3	45 ± 5	n.s.	unit/L
Total protein	5.7 ± 0.1	5.6 ± 0.1	5.6 ± 0.1	n.s.	g/dL
Albumin	4.4 ± 0.1	4.0 ± 0.1	3.9 ± 0.1*	n.s.	g/dL
Globulin	1.4 ± 0.1	1.6 ± 0.1	1.7 ±0.1	n.s.	g/dL
Amylase	664 ± 32	540 ± 17	468 ± 16*	n.s.	mg/dL
Blood urea nitrogen	14 ± 1	17 ± 1	13 ± 1	n.s.	mg/dL

- $p<0.05$ compared to vehicle treated mice by one-way ANOVA with Dunn's multiple comparison test.
- n.s.; not specified

SI Appendix Table 3. Off Target Receptor Binding of 1V270				
Target Compounds[*1]	Species	Incubation temperature (°C)	Incubation time (min)	1V270[*2]
CYP450, 1A2[*3]	human	37	**30**	3
CYP450, 2C19[*3]	human	37	45	-2
CYP450, 2C9[*3]	human	37	45	-1
CYP450, 2D6[*3]	human	37	45	2
CYP450, 3A4[*3]	human	37	30	-3
Adenosine A_1	human	25	90	-1
Adenosine A_{2A}	human	25	90	6
Adenosine A_3	human	25	60	8
Adrenergic a_{1A}	rat	25	60	7
Adrenergic a_{1B}	rat	25	60	-3
Adrenergic a_{1D}	human	25	60	18
Adrenergic a_{2A}	human	25	60	25
Adrenergic b_1	human	25	120	5
Adrenergic b2	human	25	60	11
Androgen (Testosterone) AR	rat	4	240	**99**
Bradykinin B1	human	25	60	1
Bradykinin B2	human	25	60	9
Calcium Channel L-Type, Benzothiazepine	rat	4	180	1
Calcium Channel L-Type, Dihydropyridine	rat	25	90	8
Calcium Channel N-Type	rat	4	30	-22
Cannabinoid CB1	human	37	90	-7
Dopamine D1	human	37	120	2
Dopamine D24	human	37	120	10
Dopamine D3	human	37	120	3
Dopamine D4.2	human	25	120	10
Endothelin ET_A	human	37	120	-19
Endothelin ET_B	human	25	120	12
Epidermal Growth Factor (EGF)	human	25	60	11
Estrogen ERa	human	25	120	0
$GABA_A$, Flunitrazepam, Central	rat	25	120	12
$GABA_A$, Muscimol, Central	rat	4	10	8
$GABA_{B1A}$	human	25	180	-20
Glucocorticoid	human	25	120	7
Glutamate, Kainate	rat	4	60	10
Glutamate, NMDA, Agonism	rat	4	20	1
Glutamate, NMDA, Glycine	rat	4	30	16
Glutamate, NMDA, Phencyclidine	rat	25	45	17
Histamine H_1	human	25	180	8
Histamine H_2	human	25	120	34
Histamine H_3	human	25	120	-4
Imidazoline I_2, Central	rat	25	30	23
Interleukin IL-1	mouse	37	120	2
Leukotriene, Cysteinyl CysLT1	human	25	30	4
Melatonin MT_1	human	25	180	1

Muscarinic M_1	human	25	120	6
Muscarinic M_2	human	25	120	0
Muscarinic M_3	human	25	120	2
Neuropeptide Y Y_1	human	37	60	1
Neuropeptide Y Y_2	human	37	120	-13
Nicotinic Acetylcholine	human	25	60	2
Nicotinic Acetylcholine α1, Bungarotoxin	human	25	120	0
Opiate δ1 (OP1, DOP)	human	25	60	-8
Opiate κ (OP2, KOP)	human	25	60	2
Opiate μ (OP3, MOP)	human	25	60	6
Phorbol Ester	mouse	25	60	-8
Platelet Activating Factor (PAF)	human	25	180	-8
Potassium Channel [K_{ATP}]	human	25	120	6
Potassium Channel hERG	human	25	60	-1
Prostanoid EP_4	human	25	120	3
Purinergic P_{2x}	rabbit	25	30	24
Purinergic P_{2g}	rat	25	60	17
Rolipram	rat	4	60	7
Serotonin (5-Hydroxytryptamine) 5-HT_{1A}	human	25	60	-8
Serotonin (5-Hydroxytryptamine) 5-HT_{2B}	human	37	60	-6
Serotonin (5-Hydroxytryptamine) 5-HT_3	human	37	60	-2
Sigma s1	human	37	240	12
Sodium Channel, Site 2 289593	rat	37	60	0
Tachykinin NK_1	human	4	90	7
Thyroid Hormone	rat	4	18 hrs	3
Transporter, Dopamine (DAT)	human	4	180	**2**
Transporter, GABA	rat	25	20	6
Transporter, Norepinephrine (NET)	human	4	180	**1**
Transporter, Serotonin (5-Hydroxytryptamine) (SERT)	human	25	60	5

*1; Assay were performed in duplicates. The values are average of the duplicate data.
*2; 10 μM
*3; Activities of cytochrome (CYP 450) isozyme were determined by spectrofluorimetric quantitation of 3-cyano-7-hydroxycoumarin. Other target compounds were studied using the radioligand assays.
*4:Bold letter indicates ≥ 50% inhibition.