Supplementary Text

Materials and Methods

Mice. C57BL/6 wild-type (WT) (obtained from Walter and Eliza Hall Institute, Melbourne, Australia), C57BL/6 TCRδ-deficient (TCRδ$^{-/-}$), C57BL/6 Ja281-deficient (Ja281$^{-/-}$), C57BL/6 RAG-1-deficient (RAG-1$^{-/-}$) and C57BL6-Ighm deficient (mMT) mice were bred and maintained as previously described [1, 2]. Mice 6-14 weeks of age were used in all experiments that were performed according to Peter Mac animal experimental ethics committee guidelines. Some mice were depleted of T cell subsets with anti-CD4 (GK1.5) and anti-CD8β (53.5.8) as previously described [3].

Cell lines

MALME-3M, A375, SK-MEL-2, SK-MEL-24, SK-MEL-28, WM266.4, MeWo and HS294T were obtained from the American Tissue Culture Collection and D04-M1 cells were obtained from the Queensland Institute for Medical Research. The BRAF and NRAS mutation status of these cell lines has been reported previously [4, 5]. All cell lines were maintained in RPMI 1640 containing 10% FBS, 2 mM L-alanyl-L-glutamine, 1% penicillin/streptomycin and 250 ng/ml amphotericin B in a 37°C humidified, 5% CO_2 incubator.

Statistical analysis. Data were analyzed with GraphPad Prism (version 5) software (GraphPad Software, La Jolla, CA). Significant differences between groups were assessed by a two-tailed *t* test or Mann-Whitney *U* test, as indicated. Values of $P < 0.05$ were considered significant.

References

1. Haynes NM, Hawkins ED, Li M, et al. CD11c+ dendritic cells and B cells contribute to the tumoricidal activity of anti-DR5 antibody therapy in established tumors. *J Immunol.* Jul 1 2010;185(1):532-541.

2. Paget C, Chow MT, Duret H, Mattarollo SR, Smyth MJ. Role of gammadelta T cells in alpha-galactosylceramide-mediated immunity. *J Immunol.* Apr 15 2012;188(8):3928-3939.

3. Koebel CM, Vermi W, Swann JB, et al. Adaptive immunity maintains occult cancer in an equilibrium state. *Nature.* Dec 6 2007;450(7171):903-907.

4. Ikediobi ON, Davies H, Bignell G, et al. Mutation analysis of 24 known cancer genes in the NCI-60 cell line set. *Mol Cancer Ther.* Nov 2006;5(11):2606-2612.

5. Stark M, Hayward N. Genome-wide loss of heterozygosity and copy number analysis in melanoma using high-density single-nucleotide polymorphism arrays. *Cancer Res.* Mar 15 2007;67(6):2632-2642.

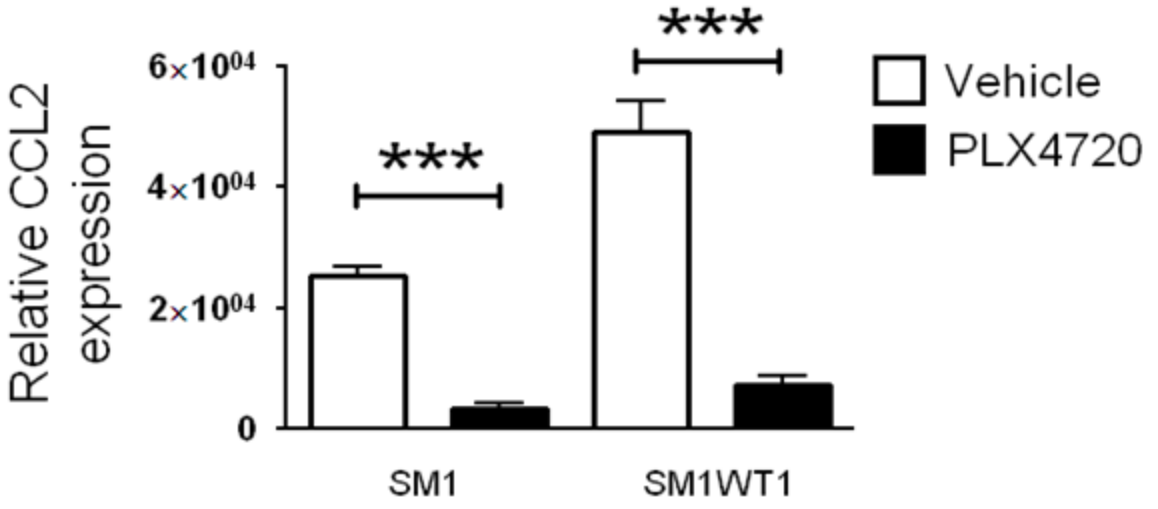
Supplementary Figures

Supplementary Table 1. PLX4720 inhibits the proliferation of human BRAF^{V600E} melanoma cells *in vitro*. To determine proliferation IC50, various human melanoma cell lines were seeded in 96-well plates and allowed to proliferate for 48 h. Cells were then treated with a range of PLX4720 concentrations (n=3). After 72 h, cell number was determined using the SRB method. IC50 concentrations were determined using non-linear regression. BRAF and NRAS status is indicated.

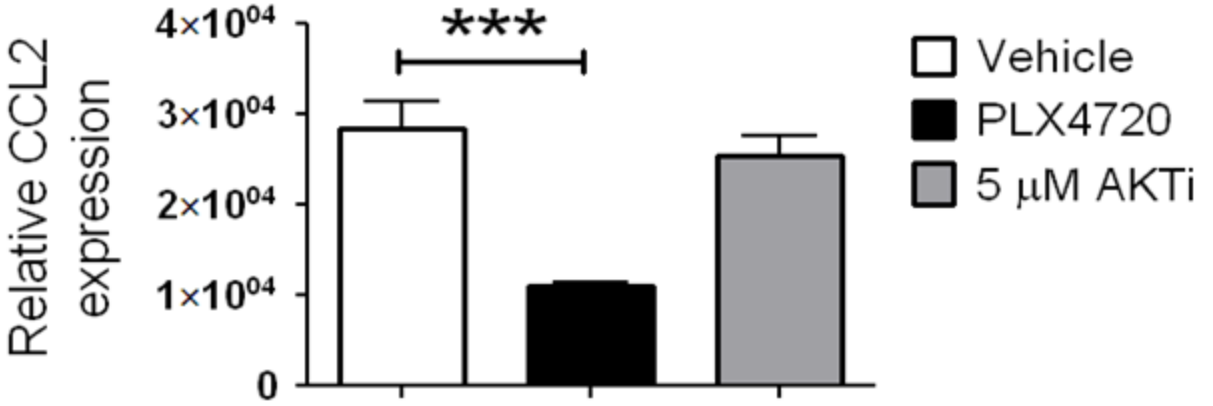
Cell line	PLX4720 IC50	BRAF status	NRAS status
MALME-3M	12.3 nM	V600E	WT
A375	16.0 nM	V600E	WT
SK-MEL-28	21 nM	V600E	WT
SK-MEL-24	42 nM	V600E	WT
WM266.4	53 nM	V600E	WT
HS294T	2 μM	V600E	WT
MeWo	7.7 μM	WT	WT
SK-MEL-2	6.9 μM	WT	Q61R
D04-M1	1.7 μM	WT	Q61L

Supplementary Fig. S1. PLX4720 suppresses tumor CCL2 expression. Relative expression of CCL2 mRNA (A) in SM1 and SM1WT1 cell lines treated with vehicle control or 10 μM PLX4720, or (B) in SM1WT1 cell line treated with vehicle, 10 μM PLX4720 or 5 μM AKT inhibitor (AKTi-1/2, Calbiochem Cat No. 124017) at 24 h. Statistical differences in relative CCL2 expression of (A) SM1 and SM1WT1 cell lines treated with vehicle or PLX4720, and (B) SM1WT1 cell line treated with vehicle, PLX4720, or AKT inhibitor for each group were determined by a unpaired t test (*** $P < 0.001$)

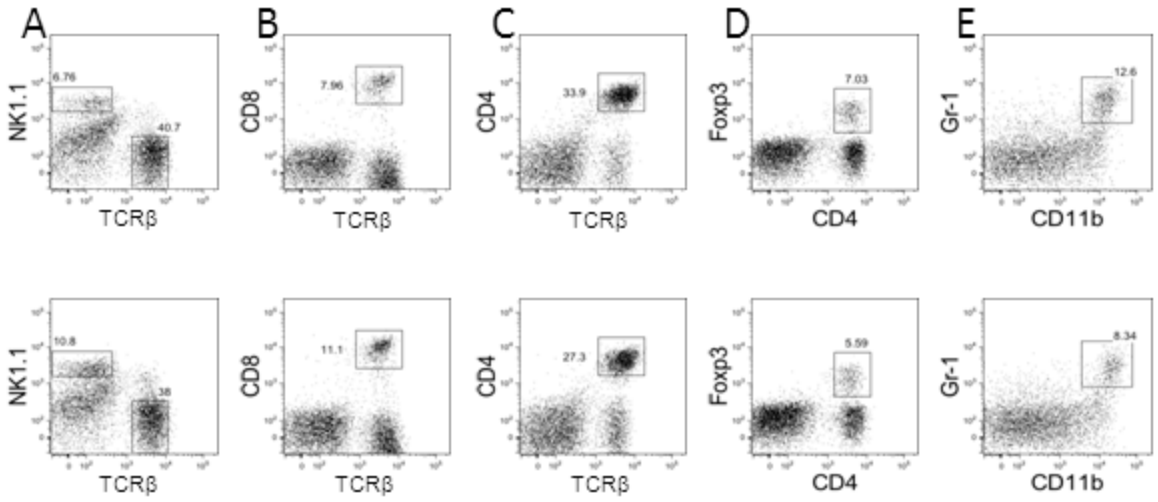
A


Relative CCL2 expression
6×10^04
4×10^04
2×10^04
0
***
***
SM1
SM1WT1
Vehicle
PLX4720

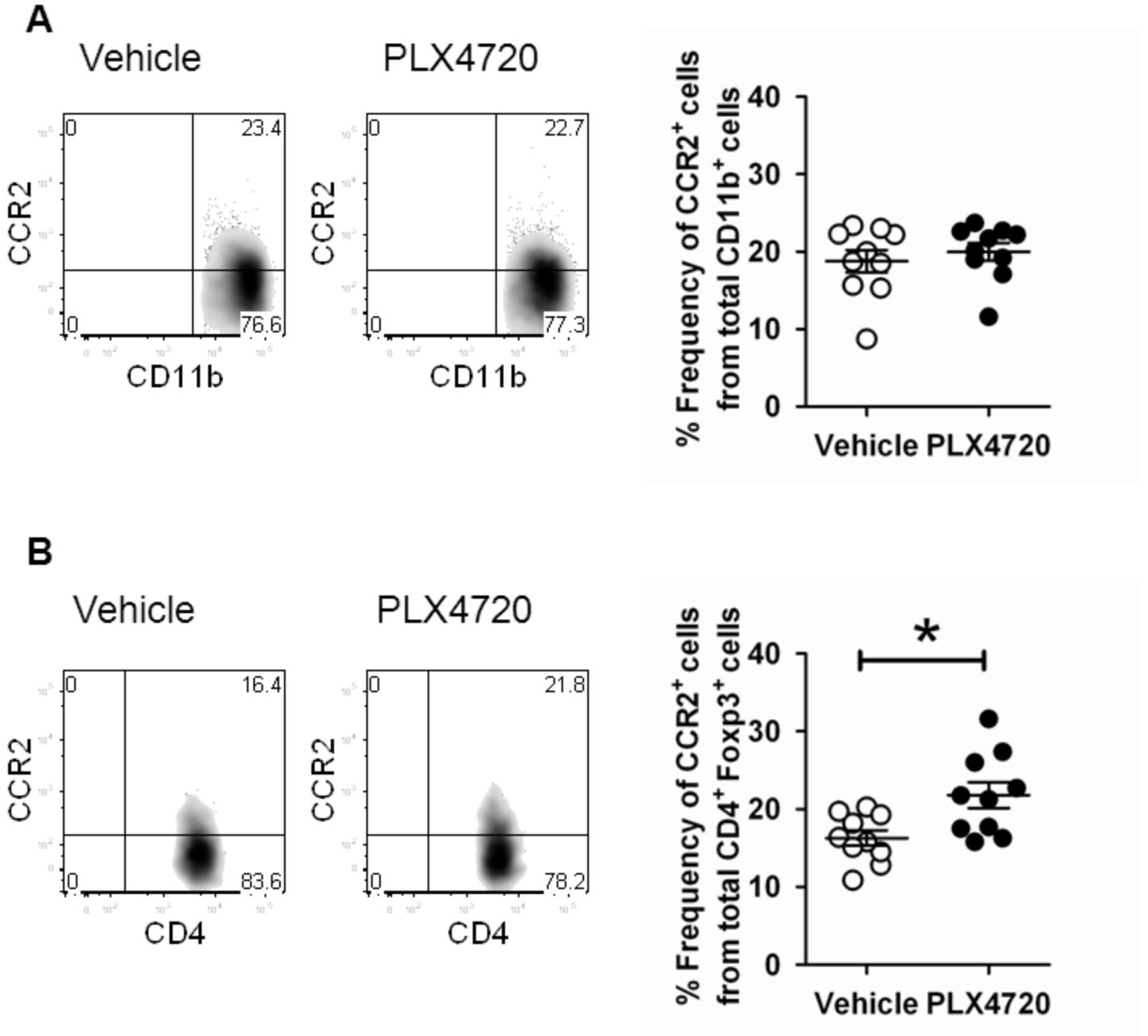
B


Relative CCL2 expression
4×10^04
3×10^04
2×10^04
1×10^04
0
***
Vehicle
PLX4720
5 μM AKTi

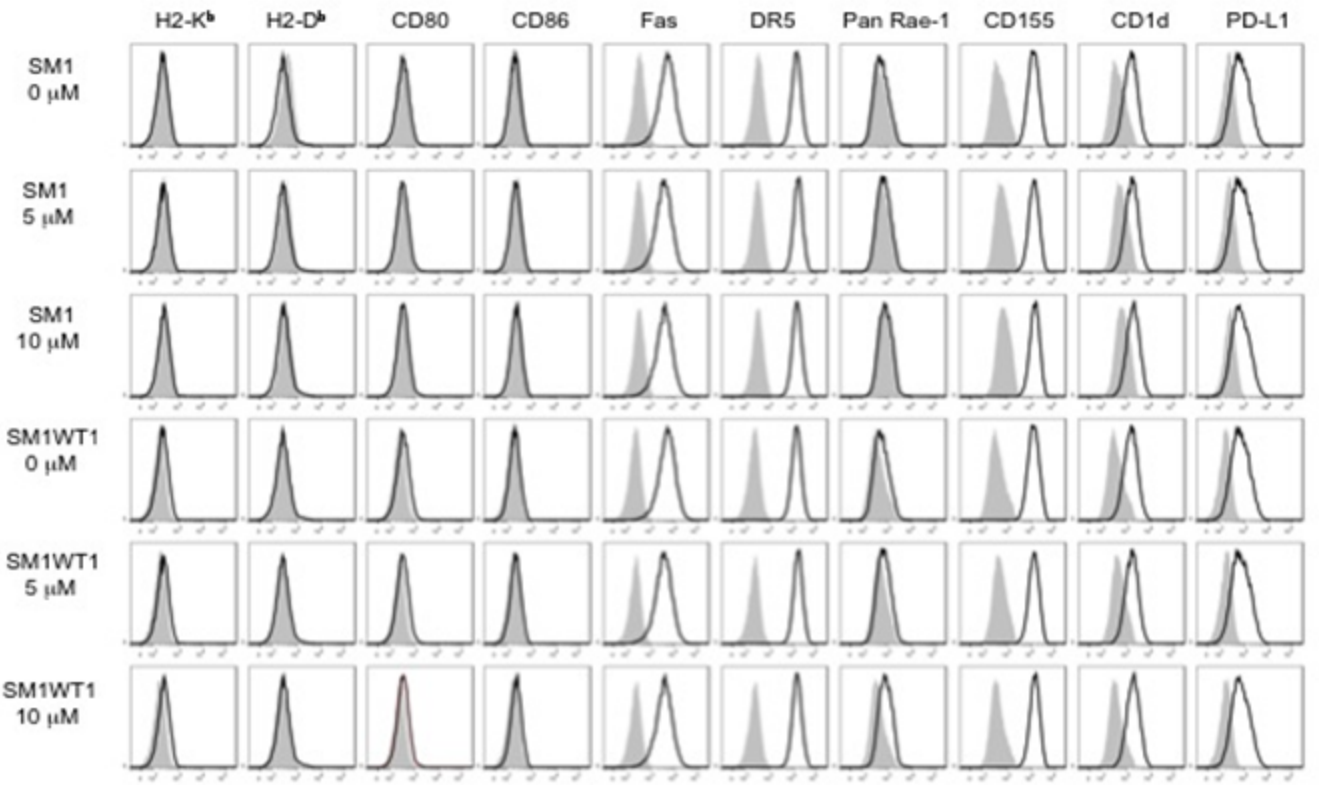
Supplementary Fig. S2. Representative flow analysis of SM1WT1 TILs post PLX4720 treatment. Groups of B6 WT mice (n=5-6) were inoculated with 1 x 10^6 SM1WT1 cells. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 12-20 post tumor inoculation. At day 21, tumors were excised and FACS analyses on TILs were performed. Representative FACS plots for frequencies of (A) NK cells and total T cells, (B) $CD8^+$ T cells, (C) $CD4^+$ T cells, (D) Tregs, (E) $CD11b^+$ $Gr\text{-}1^+$ cells, gated from (A-D) $CD45.2^+$ or (E) $CD45.2^+$ $CD3^-$ $CD19^-$ TILs from vehicle- or PLX4720-treated mice are shown.


A
B
C
D
E
NK1.1
TCRβ
6.76
40.7
CD8
7.96
CD4
33.9
Foxp3
CD4
7.03
Gr-1
CD11b
12.6
Vehicle
10.8
38
11.1
27.3
5.59
6.34
PLX4720

Supplementary Fig. S3. Intratumor CCR2 expression on CD11b$^+$ cells and FoxP3$^+$ Treg. Groups of B6 WT mice (n=10) were inoculated with 1 x 10^6 SM1WT1 cells. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 12-20 post tumor inoculation. At day 21, tumors were excised and FACS analyses on TILs were performed. Frequencies of CCR2$^+$ cells in (A) CD11b$^+$ cells, (B) CD4$^+$Foxp3$^+$ T cells TILs from vehicle- or PLX4720-treated mice are shown. Representative FACS plots for frequencies of CCR2$^+$ cells in (A) CD11b$^+$ cells, (B) CD4$^+$Foxp3$^+$ TILs from vehicle- or PLX4720- treated mice are shown.


A
Vehicle
PLX4720
CCR2
CD11b
0
23.4
76.6
22.7
77.3
% Frequency of CCR2+ cells from total CD11b+ cells
40
30
20
10
0
Vehicle PLX4720
B
Vehicle
PLX4720
CCR2
CD4
0
16.4
83.6
21.8
78.2
% Frequency of CCR2+ cells from total CD4+ Foxp3+ cells
*
40
30
20
10
0
Vehicle PLX4720

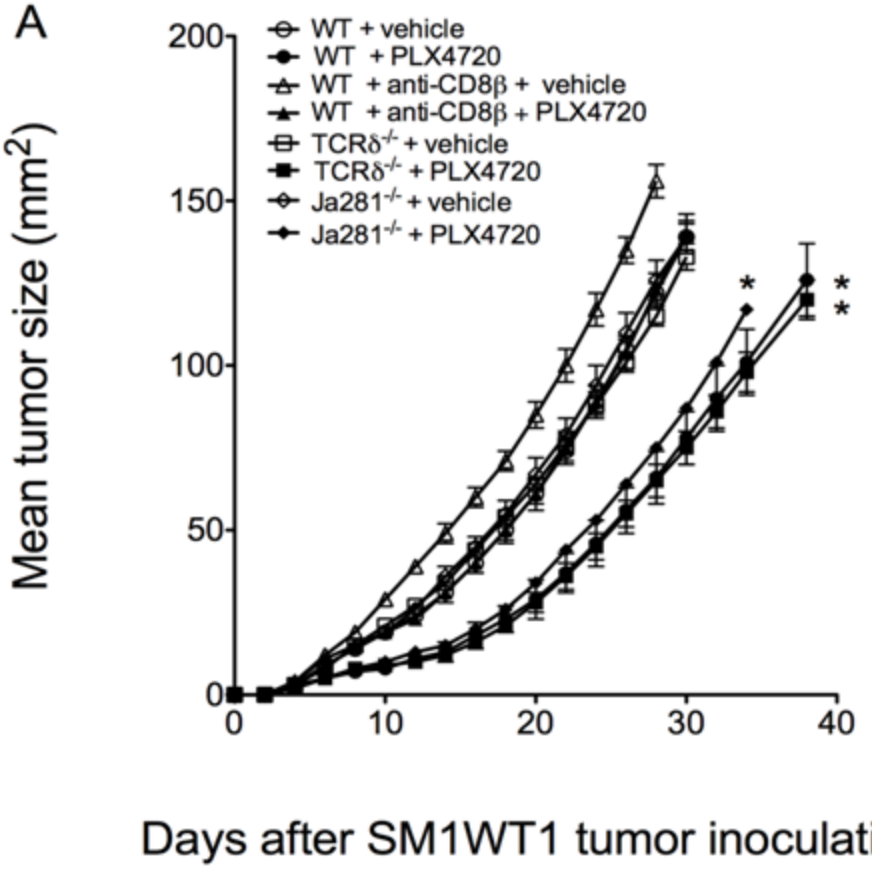
Supplementary Fig. S4. PLX4720 does not increase the immunogenicity of SM1 or SM1WT1 melanomas. SM1 and SM1WT1 cells (5 x 10^5) cells were seeded into three medium size flasks per line. Cells were incubated for 24 h, then replaced with culture media containing 12 ml 10% FCS complete RPMI plus with 0 μM (1:1000 diluted DMSO), 5.0 μM and 10 μM PLX4720. After 24 h incubation, cells were collected, washed once with FACS buffer, and stained with the isotype control (grey shaded) or corresponding mAbs as indicated (solid line). Representative of two experiments performed.


H2-K^b
H2-D^b
CD80
CD86
Fas
DR5
Pan Rae-1
CD155
CD1d
PD-L1
SM1
0 μM
SM1
5 μM
SM1
10 μM
SM1WT1
0 μM
SM1WT1
5 μM
SM1WT1
10 μM

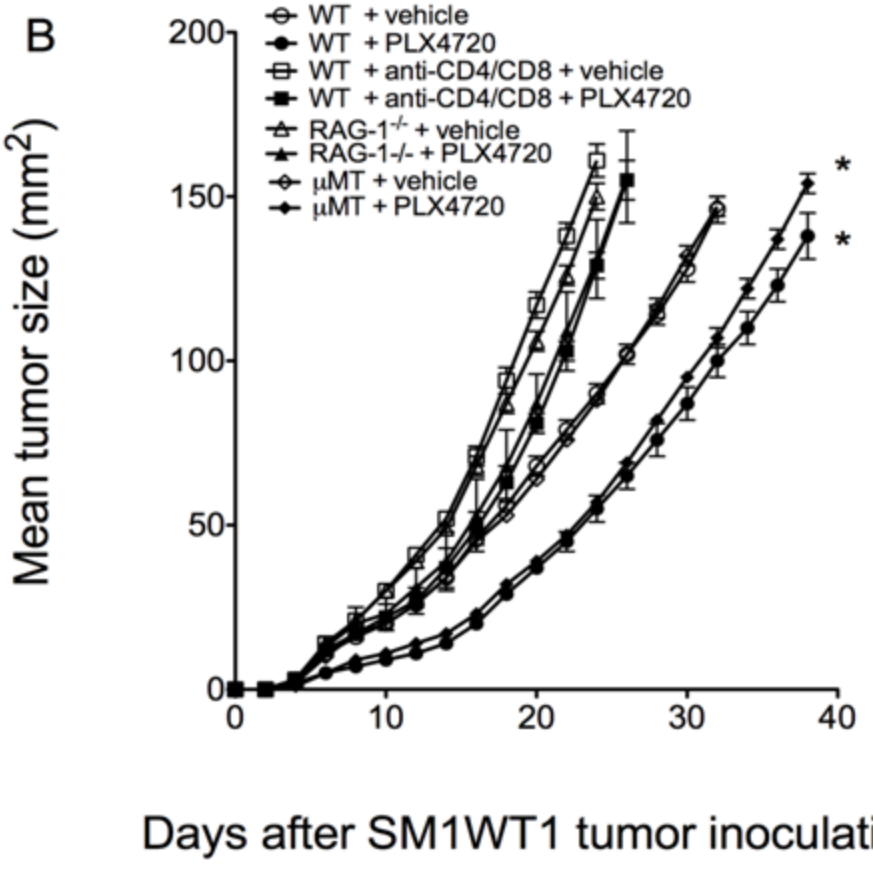
Supplementary Fig. S5. PLX4720 anti-tumor activity is $CD8^+$ T cell-dependent.

(a) Groups of 5 WT, $TCR\delta^{-/-}$ or $Ja281^{-/-}$ mice were inoculated with 5 x 10^5 SM1WT1 cells. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 3-10 post tumor inoculation. Some groups of WT mice were additionally treated with anti-CD8β (100 μg i.p.) on days 2,3,10, 17 and 24 after tumor inoculation. **(b)** Groups of 5 WT, $RAG\text{-}1^{-/-}$, and μMT mice were inoculated with 5 x 10^5 SM1WT1 cells. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 3-10 post tumor inoculation. Some groups of WT mice were additionally treated with anti-CD4 and anti-CD8β (100 μg i.p. each) on days 2,3,10, 17 and 24 after tumor inoculation. Tumor sizes are represented as the mean $\pm$ SEM. Statistical differences in tumor sizes between mice treated with vehicle versus PLX4720 for each group were determined by a Mann-Whitney test (* $P < 0.05$).

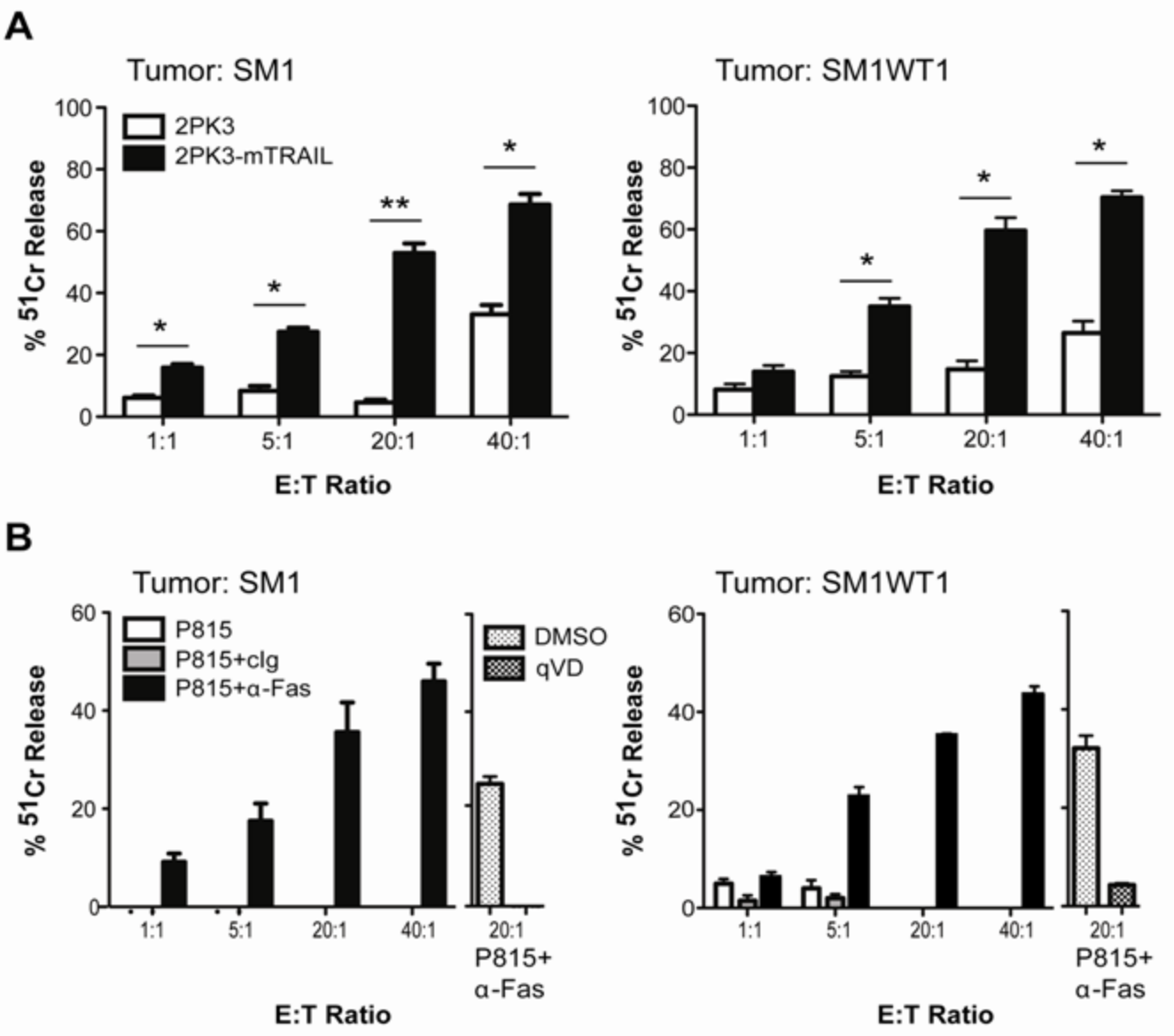
A


WT + vehicle
WT + PLX4720
WT + anti-CD8β + vehicle
WT + anti-CD8β + PLX4720
TCRδ-/- + vehicle
TCRδ-/- + PLX4720
Ja281-/- + vehicle
Ja281-/- + PLX4720
Mean tumor size (mm2)
Days after SM1WT1 tumor inoculation
0
50
100
150
200
0
10
20
30
40
*
*

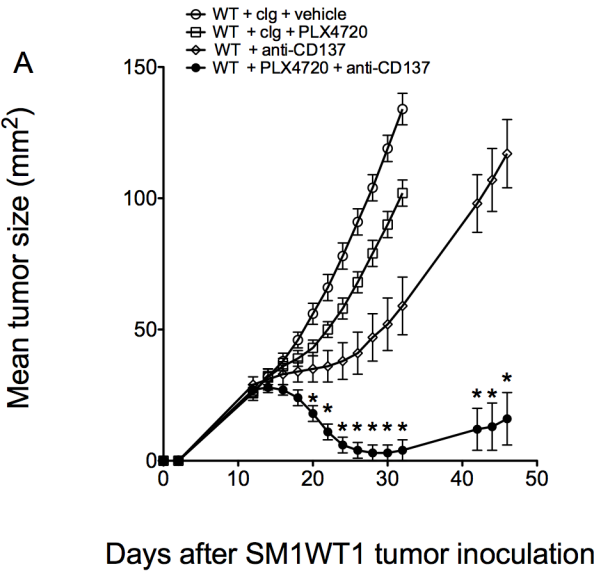
B

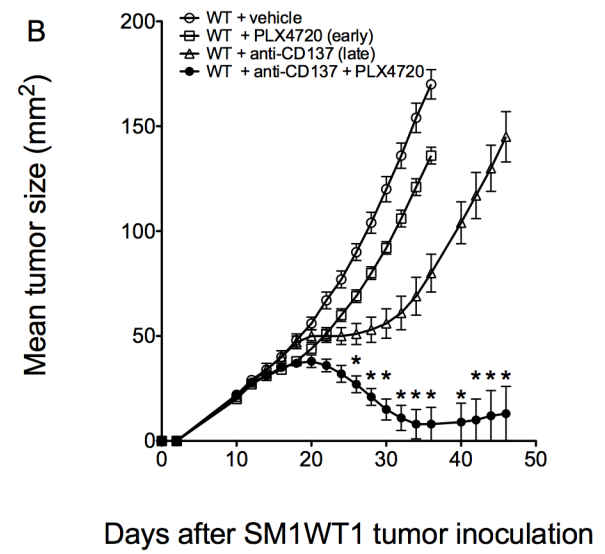

WT + vehicle
WT + PLX4720
WT + anti-CD4/CD8 + vehicle
WT + anti-CD4/CD8 + PLX4720
RAG-1-/- + vehicle
RAG-1-/- + PLX4720
μMT + vehicle
μMT + PLX4720
Mean tumor size (mm2)
Days after SM1WT1 tumor inoculation
0
50
100
150
200
0
10
20
30
40
*
*

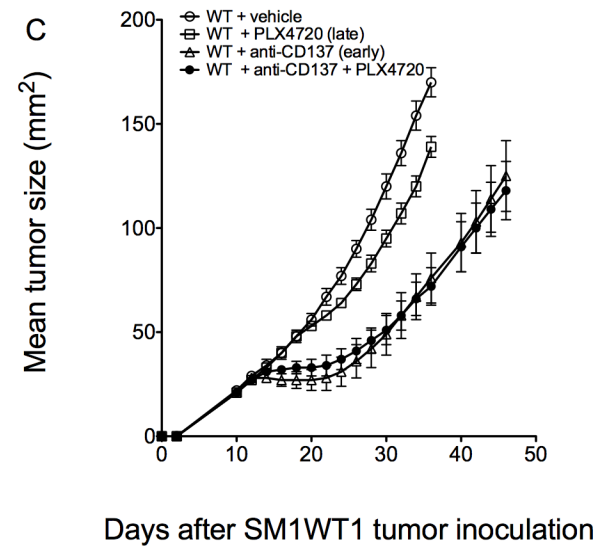
Supplementary Fig. S6. Sensitivity of the SM1 and SM1-WT1 melanoma cell lines to TRAIL and Fas mediated apoptosis. Twenty hour ^{51}Cr release assays were used to assess the sensitivity of the SM1 and SM1WT1 tumor lines to 2PK-3-mTRAIL effectors (A) or an anti-Fas (CD95; BD Pharmingen) antibody (10 μg/ml) cross-linked by FcR^+ P815 effectors (B) at the indicated effector/target tumor cell ratios (solid bars). Levels of ^{51}Cr detection in control wells containing mock-transfected 2PK3 cells (A) or UC8-1B9 control Ig (cIg) (B) are shown (white bars). The apoptotic activity of the anti-Fas antibody was abrogated by pre-treatment of the SM1 and SM1WT1 cells with the pan-caspase inhibitor, qVD (10 μmol/L, InSolution Q-VD-OPh; Calbiochem) (B). DMSO treatment did not disrupt the apoptotic effects of the α-Fas antibody. Each group was performed in triplicate. Results are representative of two experiments (n=6). Statistical differences in ^{51}Cr release between the 2PK3-mTRAIL and 2PK3 treated SM1 and SM1-WT1 tumor lines were determined by the Mann-Whitney *U* test. *, $P<0.003$ and **$P<0.0001$.


A
Tumor: SM1
2PK3
2PK3-mTRAIL
% 51Cr Release
E:T Ratio
1:1
5:1
20:1
40:1
Tumor: SM1WT1
B
Tumor: SM1
P815
P815+clg
P815+α-Fas
DMSO
qVD
P815+
α-Fas
Tumor: SM1WT1

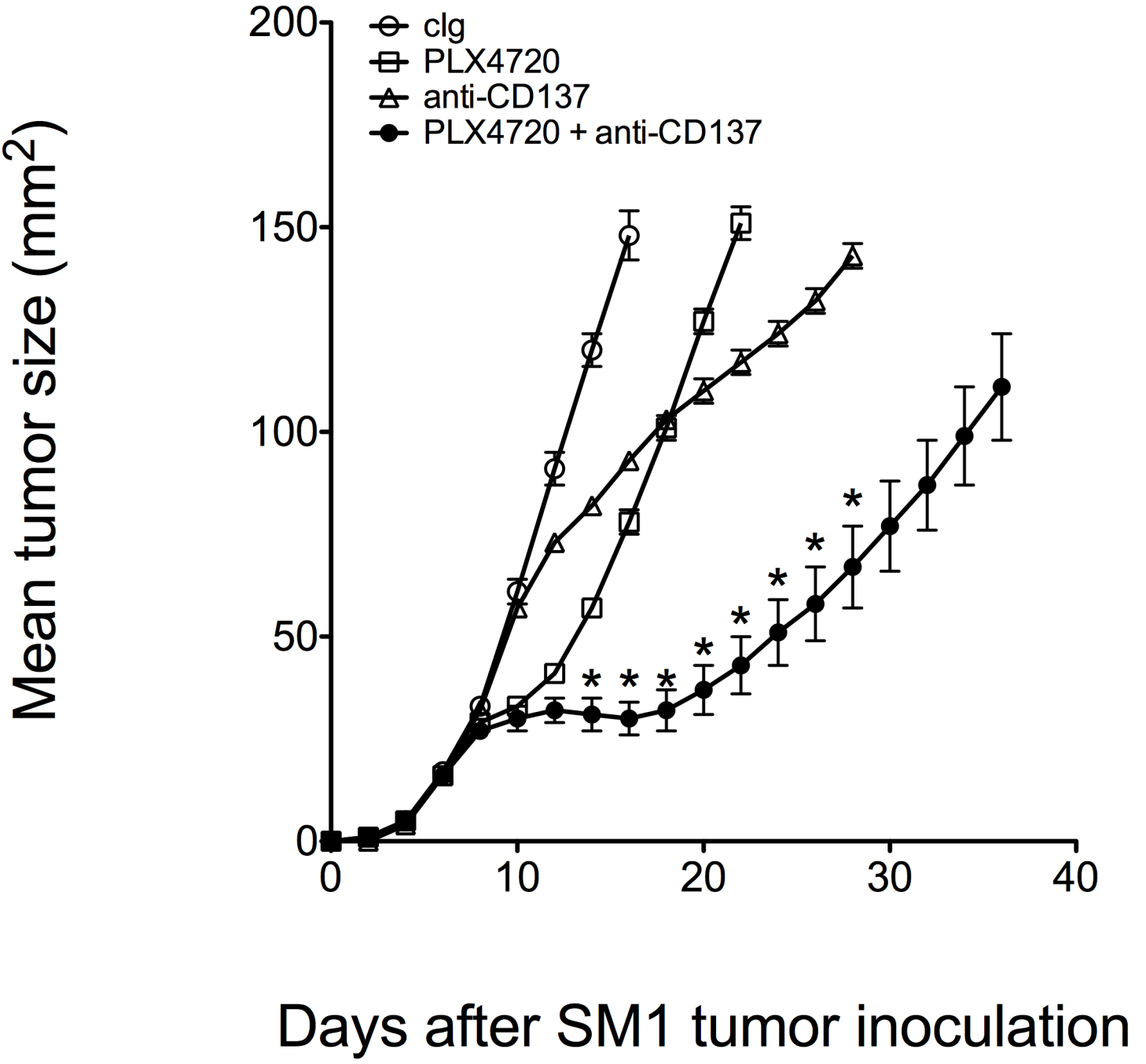
Supplementary Fig. S7. Anti-tumor activity of coincident PLX4720 and anti-CD137 in combination. Groups of 5 WT mice were inoculated with 5 x 10^5 SM1WT1 cells. Mice received **(A)** vehicle or PLX4720 (20 mg/kg i.p.) daily from day 12-16 post tumor inoculation. Some groups of mice were additionally treated with cIg or anti-CD137 (100 μg i.p. each) on days 12, 14, 16 and 18 after tumor inoculation. (B) vehicle or PLX4720 (20 mg/kg i.p.) daily from day 12-16 post tumor inoculation (early). Some groups of mice were additionally treated with cIg or anti-CD137 (100 μg i.p. each) on days 17, 19, and 21 after tumor inoculation (late). (C) vehicle or PLX4720 (20 mg/kg i.p.) daily from day 17-21 post tumor inoculation (late). Some groups of mice were additionally treated with cIg or anti-CD137 (100 μg i.p. each) on days 12, 14, and 16 after tumor inoculation (early). Tumor sizes are represented as the mean $\pm$ SEM. Statistical differences in tumor sizes between mice treated with anti-CD137 alone versus combination therapy were determined by a Mann-Whitney test (* $P < 0.05$).


A
WT + clg + vehicle
WT + clg + PLX4720
WT + anti-CD137
WT + PLX4720 + anti-CD137
Mean tumor size (mm2)
150
100
50
0
0
10
20
30
40
50
Days after SM1WT1 tumor inoculation

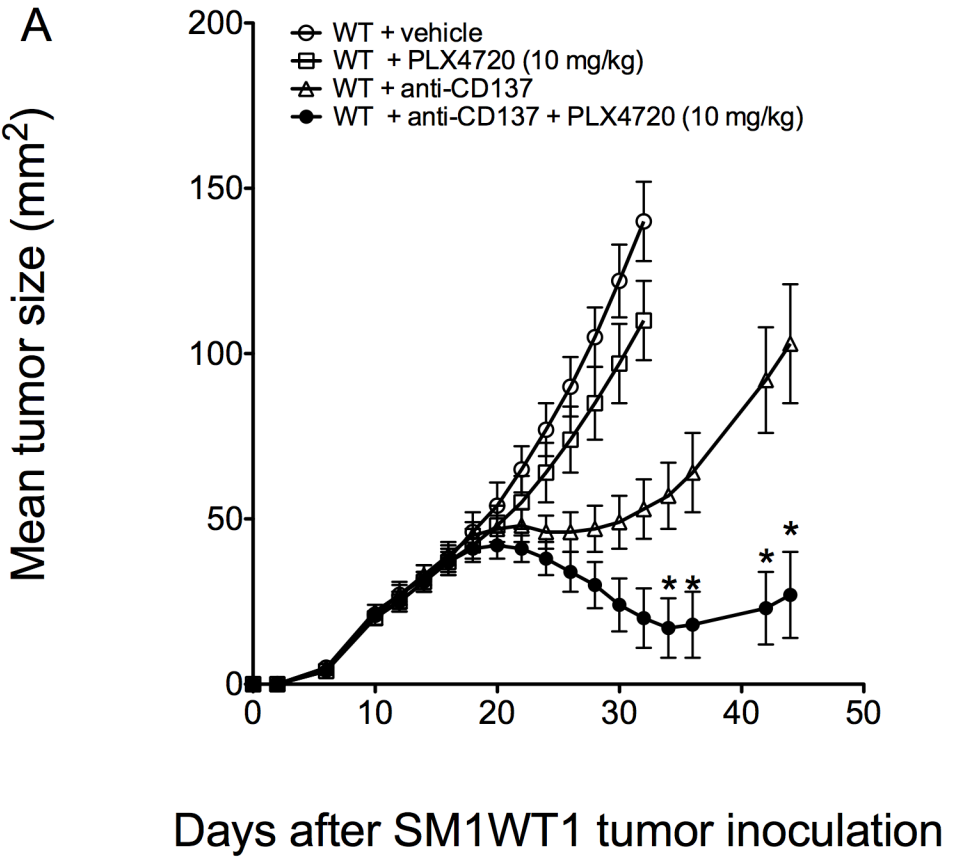

B
WT + vehicle
WT + PLX4720 (early)
WT + anti-CD137 (late)
WT + anti-CD137 + PLX4720
Mean tumor size (mm2)
200
150
100
50
0
0
10
20
30
40
50
Days after SM1WT1 tumor inoculation

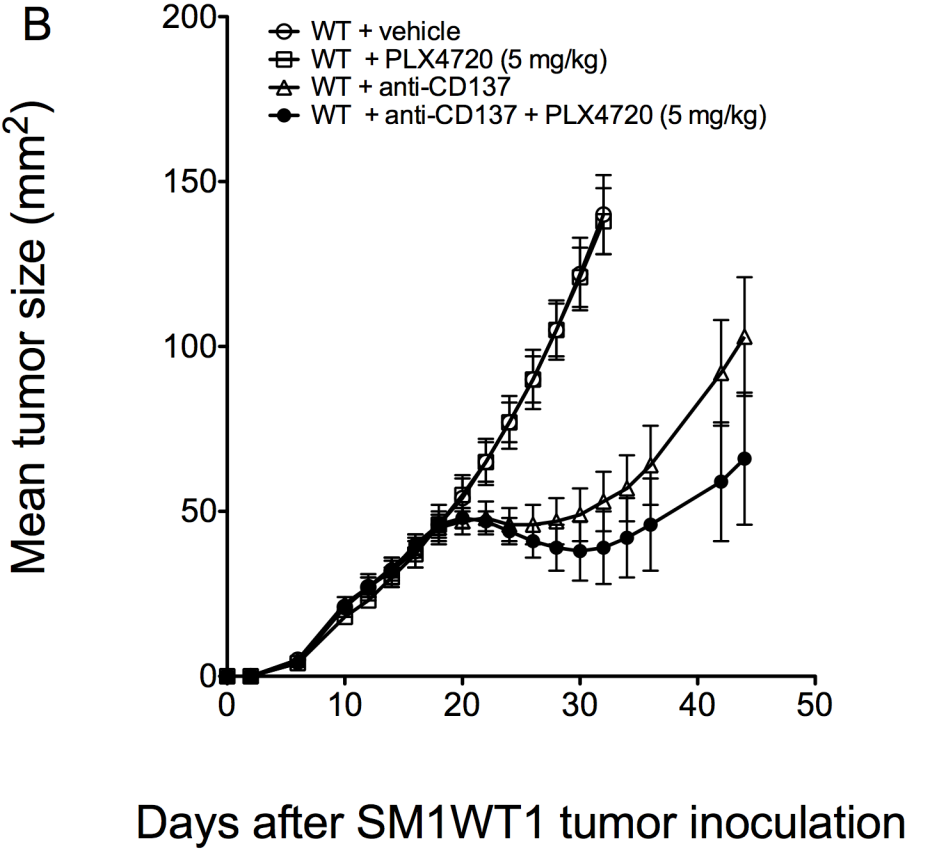

C
WT + vehicle
WT + PLX4720 (late)
WT + anti-CD137 (early)
WT + anti-CD137 + PLX4720
Mean tumor size (mm2)
200
150
100
50
0
0
10
20
30
40
50
Days after SM1WT1 tumor inoculation

Supplementary Fig. S8. Synergistic anti-tumor activity of PLX4720 and anti-CD137. Groups of 5 WT mice were inoculated with 5 x 10^5 SM1 cells. Mice received vehicle or PLX4720 (20 mg/kg i.p.) daily from day 7-10 after tumor inoculation and cIg or anti-CD137 (100 μg i.p.) on days 11, 12, 13 and 14 after tumor inoculation. Tumor sizes are represented as the mean ± SEM. Statistical differences in tumor sizes between mice treated with single versus combination therapy were determined by a Mann-Whitney test (* $P < 0.05$).


clg
PLX4720
anti-CD137
PLX4720 + anti-CD137
Mean tumor size (mm2)
Days after SM1 tumor inoculation
0
50
100
150
200
0
10
20
30
40
*

Supplementary Fig. S9. PLX4720 dose effect on the anti-tumor activity of the PLX4720 and anti-CD137 combination. Groups of 5 WT mice were inoculated with 5 x 10^5 SM1WT1 cells. Mice received **(A)** vehicle or PLX4720 (10 mg/kg i.p.) or (B) vehicle or PLX4720 (5 mg/kg i.p.) daily from day 12-16 post tumor inoculation. Some groups of mice were additionally treated with cIg or anti-CD137 (100 μg i.p. each) on days 17, 19, 21 and 23 after tumor inoculation. Tumor sizes are represented as the mean $\pm$ SEM. Statistical differences in tumor sizes between mice treated with anti-CD137 alone versus combination therapy were determined by a Mann-Whitney test (* $P < 0.05$).


A
WT + vehicle
WT + PLX4720 (10 mg/kg)
WT + anti-CD137
WT + anti-CD137 + PLX4720 (10 mg/kg)
Mean tumor size (mm2)
200
150
100
50
0
0
10
20
30
40
50
*
*
*
Days after SM1WT1 tumor inoculation


B
WT + vehicle
WT + PLX4720 (5 mg/kg)
WT + anti-CD137
WT + anti-CD137 + PLX4720 (5 mg/kg)
Mean tumor size (mm2)
200
150
100
50
0
0
10
20
30
40
50
Days after SM1WT1 tumor inoculation