Supplemental Figure Legends

Supplemental Figure 1: Dose intensification impacts tumor growth in multiple tumor types. Final tumor measurements of A) HCT116 p53-/- B) HT29 C) HT-29 xenograft-bearing mice treated with 50 mg/kg ONC201 dosed at indicated frequencies and D) Single mouse images of HT29 xenografts from mice treated as shown. E) MRI of 100 mg/kg per 1 week and vehicle after 8 wks. (N=6 in HT29 and HCT116 p53-null, N=4 in MDA-MB-231).

Supplemental Figure 2: Oral vs IP administration, and weekly vs daily dosing of ONC 201 have comparable effects. A) Final relative tumor growth of HT29 xenografts treated as shown B) Tumor growth of HT29 in mice treated as indicated for 100 mg/kg ONC201 treatments (Twice a week was on a Monday/Thursday schedule) and C) Final percent tumor growth of these cohorts. (For mouse studies, N=4 for each). (P values are as indicated: ** < 0.01, *** < 0.001 compared to the vehicle unless indicated using 2-side Wilcoxon rank sum test).

Supplemental Figure 3: Increased ONC201 dose and frequency does not increase toxicity. A) Pathology of athymic nude mice treated with 100 mg/kg weekly vs vehicle. No lesions noted. N=3. B) Toxicology chemistry serum panel of indicated cohorts in athymic nude mice. Toxicology results highlighted. C) Weight of HT29 xenografts from 100 mg/kg cohorts. (N=6 for HCT116 and HT29 studies).

Supplemental Figure 4: Expression of TRAIL, ATF4, and CHOP increase as a result of increased frequency of ONC201 administration. A) ATF4 and CHOP mRNA expression in xenografts from mice treated as shown. B) TRAIL standard curve and controls. Positive control: spiked serum to 100 pg/ml. TRAIL serum levels shown correlated to (top): frequency, and (bottom): dose. C) Serum TRAIL levels in MDA-MB-231 xenograft mice at indicated doses and frequencies. D) TRAIL IHC analyses in mice treated with indicated ONC201 dose and frequency. All samples were harvested 4 weeks after

treatment began unless indicated. (For qRTPCR: N=6, ran twice in triplicate of each sample. For ELISA, n=4 ran in duplicate, samples were frozen until end of assay and ran through ELISA) (P values are as indicated: * < 0.05, ** < 0.01 compared to the vehicle unless indicated using 2-side Wilcoxon rank sum test).

Supplemental Figure 5: HT29, CT26, and MDA-MB-231 xenograft models are metastatic and ONC201 suppresses metastasis *in vivo*. A) Bioluminescence and gross imaging of HT29 metastasis in the abdomen. B) Final volume of HT29 primary tumors and metastases in mice treated as indicated. C) Total metastatic tumors (micro or bulk), measured size, and internal locations in HT29-xenograft-bearing mice of described cohorts. D) Bioluminescence imaging of HT29 xenograft-bearing mouse liver and lungs treated at two different frequencies of ONC201 administration at a dose of 25 mg/kg E) MRI of vehicle-treated mice with HT29 xenografts shows 4 tumors. F) Gross, bioluminescent, and histological imaging of metastatic tumors of MDA-MB-231 xenografts. G) CT26 lung metastases observed in the vehicle-treated mice and total number of metastases seen in each cohort. H) Estimated size of HCT116-tail vein treated mice before and after treatment with vehicle and/or ONC201. I) Number of confirmed metastases in mice that were treated with Vehicle and/or ONC201 24 hours after tail vein injection of HCT116 xenograft samples. (N=10 in CT26, N=6 in HT29, N=4 in MDA-MB-231, N=5 in HCT116 tail vein injected mice). All colorectal xenograft samples were harvested 4 weeks after treatment began unless indicated, MDA-MB-231 was harvested after 6 weeks of treatment. (P value as indicated: * < 0.05 compared to the vehicle unless indicated using 2-side Wilcoxon rank sum test, for h: difference between post/pre-treatment compared using 2-side Wilcoxon rank sum test).

Supplemental Figure 6: ONC201 suppresses migration and invasion *in vitro*. A) Xcelligence invasion kinetics assay using 1:40 matrigel and HCT116 p53-/- cells. Number of cells migrated in Boyden chamber assay after 48 hr of described cohorts in B) HCT116 p53-/-, (C) HCT116 *Bax*$^{-/-}$ ONC201-apoptosis resistant cells, and (D) HT-29 cells by boyden assay after 48 hr. E) Boyden cell

migration of MDA-MB-231 ONC201- and RIK2-treated cells. F) Scratch assay of MDA-MB-231 cells before treatment and 48 hr after treatment. G) Number of metastases per mouse of MDA-MB-231 wildtype or shTRAIL injected through tail vein injection and treated with Vehicle or ONC201 immediately after. H) Representative lung tumor images. I) Lung H&E images from tail vein mice. (For in vitro migration/invasion studies, N=4 ran two separate times. For mouse tumor studies, N=5 for MDA-MB-231 and MDA-MB231 shTRAIL. All samples were harvested 3 weeks after treatment began unless indicated). (P value as indicated: ** < 0.01 compared to the vehicle unless indicated using 2-side Wilcoxon rank sum test. For g, metastases were grouped '<2' and '$\geq$2' for the comparison between wildtype and shTRAIL ONC201 groups using 2-sided Fisher's exact test, there was no significant difference).

Supplemental Figure 7: Schematic of gating procedure for NK/T cells and activated NK cells. Singlets followed by lymphocytes where gated for vehicle tumors, ONC201 treated tumors, spleen samples, and PBMC. There was a larger volume of infiltrating lymphocytes in ONC201 vs Vehicle so there were more cells analyzed when lymphocytes where gated. A) Lymphocytes where analyzed by CD45 vs CD19; and CD45+/CD19- (non-B cell CD45 population) was selected. Cells where then analyzed NK vs CD3. B) Lymphocytes where analyzed by CD45 vs CD19; and CD45+/CD19- (non-B cell CD45 population) was selected. NK+ cells where then selected by side scatter and activated NK cells were analyzed using granzyme.

Supplemental Figure 8: Schematic of gating procedure for T cell recruitment and MDSC. Singlets where gated for vehicle tumors, ONC201 treated tumors, spleen samples, and PBMC. A) Lymphocytes where selected, there were a larger volume of infiltrating lymphocytes in ONC201 vs Vehicle so there were more cells analyzed when lymphocytes where gated. Lymphocytes where analyzed by CD45 vs CD19; and CD45+/CD19- (non-B cell CD45 population) was selected. CD3+ cells where then selected by side scatter and cells were analyzed by CD4 vs CD8. B) Lymphocytes where selected, followed by PI

staining, CD45 vs CD19; and the CD45+/CD19- (non-B cell CD45 population) was selected. CD3 and NKp46 where individually selected and then PD-1 staining assessed.

Supplemental Figure 9: ONC201 is efficacious in syngeneic models and has increased NK cells and T cells in both tumor bearing and non-tumor bearing mice. A) Tumor volume over time of MC38 xenografts in wildtype C57/BL6 mice. B) Quantitation of GFP+ cells in tumor sections in NCR1-GFP mice bearing MC38 xenografts after 4 weeks. C) Analysis of lymphocytes, NK cells, CD3 cells, and CD3+ cell differentiation in MC38 xenografts in NCR1-GFP mice after 4 weeks. D) Immunohistochemistry of MC38 tumors in NCR1-GFP C57/BL6 mice of GFP-expressing tumors (top) and CD3+ cells (bottom). E) % of CD3+ cells and NKp46+ Cells within the singlet, leukocytes, PI-, CD45+ population of PBMC in C57/BL6 non-tumor bearing mice. F) Percent IFNγ+ NK cells within the MC38-GFP mice in lymphocyte CD45+ CD19- NK+ cell population in vehicle and ONC201 treated mice. G) Analysis of T-cells in MC38 tumors in NCR1-gfp mice. Analysis of T cells, NK cells, granzyme, and CD4/CD8 expression on tumors was performed on lymphocytes, which where greater by ONC201 treatments, then CD45+/CD19- population. All treatments where with vehicle or 100mg/kg ONC201 weekly delivered orally. (For mouse studies, N=6 in wildtype C57/BL6 study. N=4 in NCR1-GFP study. N=7 in Balb/c study, for immunofluorescence studies, 4 IF slides where analyzed per tumor). (P value as indicated: * $p < 0.01$, **** $p < 0.001$ using 2-side Wilcoxon rank sum test; for GFP IF studies when 4 IF slides were analyzed per tumor, the mean of each tumor were compared using a 2-sided Wilcoxon rank sum test).

Supplemental Figure 10: Primary NK cells from healthy donors are activated by ONC201. A) Purified primary NK cells schematic for IFNγ assay. B) Schematic for Lamp1+ assay. C) Results of a second patient on IFNγ expression in increasing doses of ONC201. D) LAMP1+ expression of 5 patients with vehicle or ONC201. (N=4) (P value as indicated: * $p < 0.01$ using 2-side Wilcoxon rank sum test).

Supplemental Figure 11: NK cells play a role efficacy of ONC201 in vivo. A) Tumor volume after 5 weeks in RKO-ONC201 resistant tumor types. B) Final tumor volume after 4 weeks in ONC201 treated MC38 bearing C57/BL6 mice treated with Vehicle, ONC201 and/or GM-1. C) Representative image of ONC201 and GM1 study. D) Final tumor volume after 4 weeks in ONC201 treated MC38 bearing C57/BL6 mice or C57/BL6 Perforin knockout (*Prf*$^{-/-}$) mice treated with Vehicle or ONC201. E) Representative image of ONC201 Perforin-/- study. F) Final tumor volume after 4 weeks in ONC201 treated MC38 bearing C57/BL6 mice treated with Vehicle, ONC201 and/or CD-8 inhibitor. G) Representative image of ONC201 and CD-8 inhibitor study. All treatments where of vehicle and 100mg/kg ONC201 weekly delivered orally. GM-1 delivered every 5 days i.p. CD8 twice weekly 400ug i.p. (For mouse studies, RKOTR N=9 except GM-1 cohort which was N=5, all MC38 studies N=5). (P value as indicated: * $p < 0.01$ using 2-side Wilcoxon rank sum test).

Supplemental Figure 12: Immune infiltrates where not seen in lung metastases. NK and CD3+ population within the lymphocyte CD45+/CD19- populations in A) HCT116 lung metastases B) HCT116 *Bax*$^{-/-}$ lung metastases C) MC38 lung metastases D) Spleen controls from each mouse experiment. ONC201 treatment 100 mg/kg weekly dosed orally. For mouse studies, N=5 for all experiments.

Supplemental Figure 13: ONC201's efficacy is both from intrinsic cell death and NK-related. A) Final tumor volumes in athymic nude mice injected with HCT116 *Bax*$^{-/-}$ or wildtype xenografts treated for 4 weeks with Vehicle, ONC201, and/or GM-1. B) Representative images. C) Final tumor volume in NSG mice injected with HCT116 *Bax*$^{-/-}$ xenografts treated with Vehicle or ONC201. D) Representative images. E) Quantification of Ki67+ cells from IHC staining of tumor samples from mice in supplemental figure 13. F) Quantification of cleaved caspase-3 + xenografts from IHC staining of tumor samples from mice supplemental figure 13. G) Representative images. All treatments where of vehicle

and 100 mg/kg ONC201 weekly delivered orally. GM-1 delivered every 5 days i.p. (For mouse studies, N=6). Tumor sample sections are total quantification of entire slide, 2 slides per tumor imaged using Vectra software. (P values: * $p < 0.05$, *** $p < 0.005$ using 2-side Wilcoxon rank sum test in respect to vehicle, for IHC studies when 2 IHC slides were analyzed per tumor, the mean of each tumor were compared using a 2-sided Wilcoxon rank sum test).

Supplemental Figure 14: ONC201 and PD-1 in combination may be beneficial. A) Final tumor volume after 4 weeks of MC38 tumors treated with Vehicle, 25 mg/kg ONC201, and/or PD-1. B) Final tumor volume after 4 weeks of MC38 tumors treated with Vehicle, 50 mg/kg ONC201, and/or PD-1. C) Final tumor volume after 4 weeks of MC38 tumors treated with Vehicle, 100 mg/kg ONC201, and/or PD-1. D) PD-1 flow cytometry analysis of PD-1 expression on T cells in CT26 tumors. E) Example of analysis of PD-1 and PD-1 PE staining on a ONC201 tumor. F) Final tumor volumes of MC38 bearing mice treated with Vehicle, ONC201, GM-1, and/or PD-1 including ONC201 + PD-1 + GM1 G) Representative images. All treatments where of vehicle and 100 mg/kg ONC201 weekly delivered orally unless specified. GM-1 delivered every 5 days and PD-1 200 ug every 3 days. (For mouse studies: CT26 PD1 N=7. MC38 PD-1 N=6). (P value as indicated: * $p < 0.05$ using 2-side Wilcoxon rank sum test).

Supplemental Figure 15: Multiplex analysis of pro-immune factors and chemokines upregulated by ONC201. P value as indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ using 2-side Wilcoxon rank sum test, three separate conditioned media samples per cohort where harvested and ran in duplicate. The mean of each duplicate was taken and compared using a 2-sided Wilcoxon rank sum test.

Supplemental Figure 16: NK92 cells are activated and secrete TRAIL following treatment with ONC201. A) After 24 hr of 10 μM treatment of ONC201 and co-cultured in the presence or absence of A) HCT116p53-/- cells or B) MDA-MB-231 cells, NK92 cells express TRAIL and activation markers by flow cytometry. C) Co-culture results Live:Dead ratio of HCT116 *Bax*$^{-/-}$ D) Effects on HCT116 tumor cell viability based on CTG assay of co-culture with described cohorts, NK cells where aspirated

before cell titer glo was added. E) Cell viability of CTG results. (N=3, co-cultures ran in triplicate twice). (P value as indicated: * < 0.05, ** < 0.01, *** p < 0.001 compared to the vehicle, the mean of each duplicate were taken and compared using a 2-sided Wilcoxon rank sum test.)

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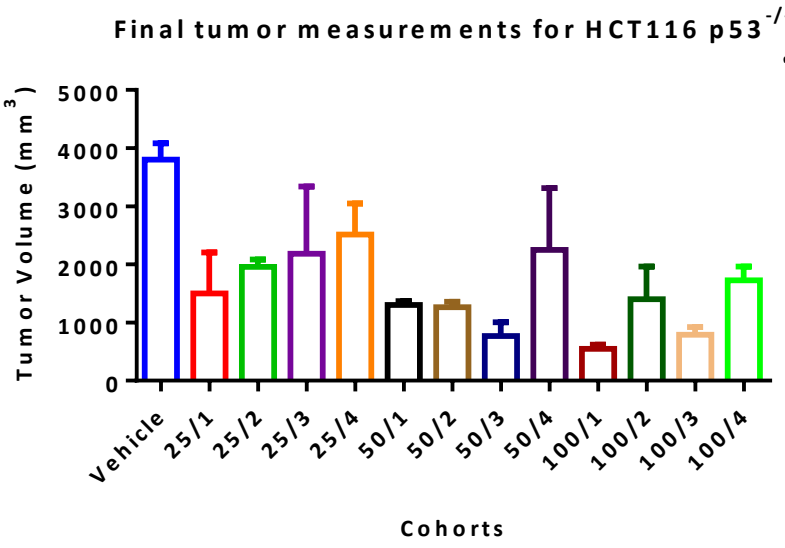

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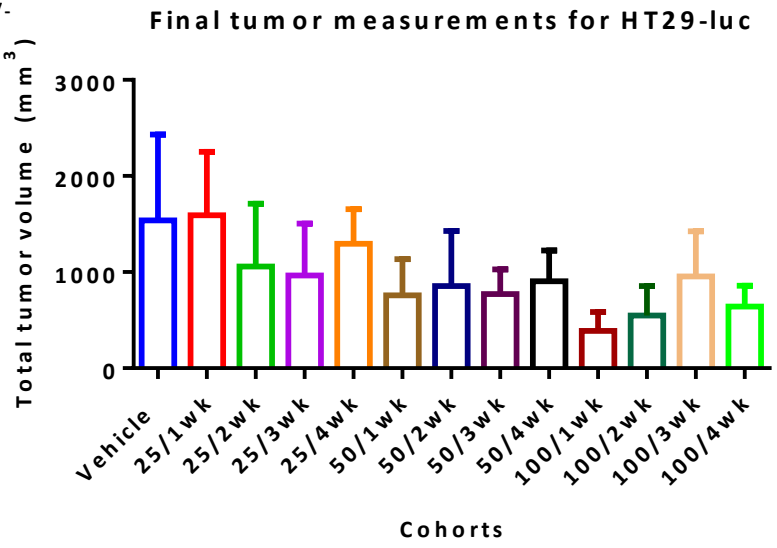

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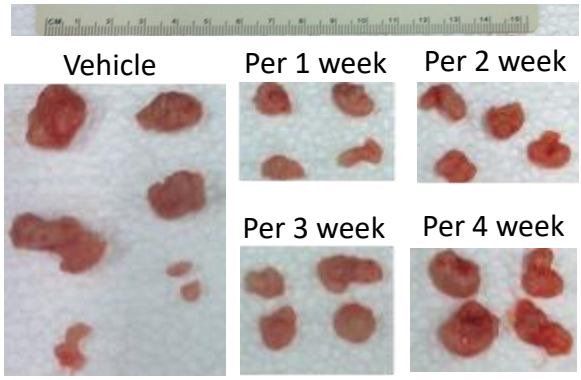

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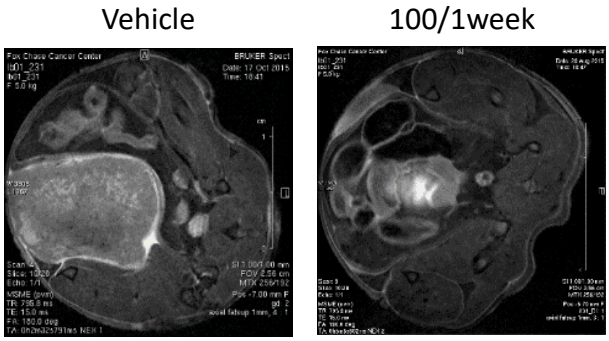

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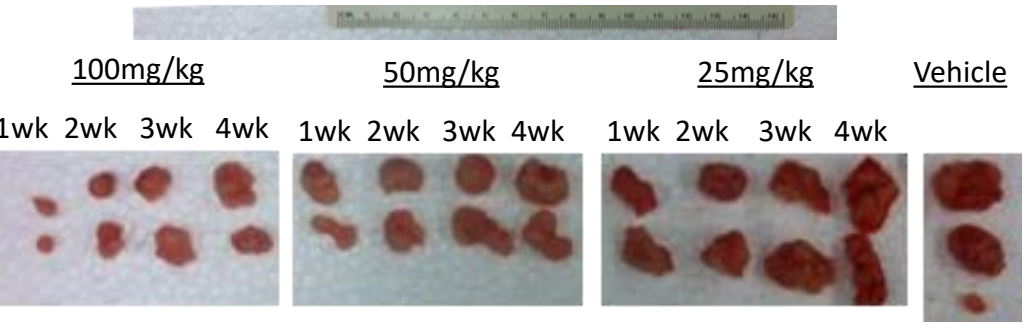

Wagner et al., Figure S1

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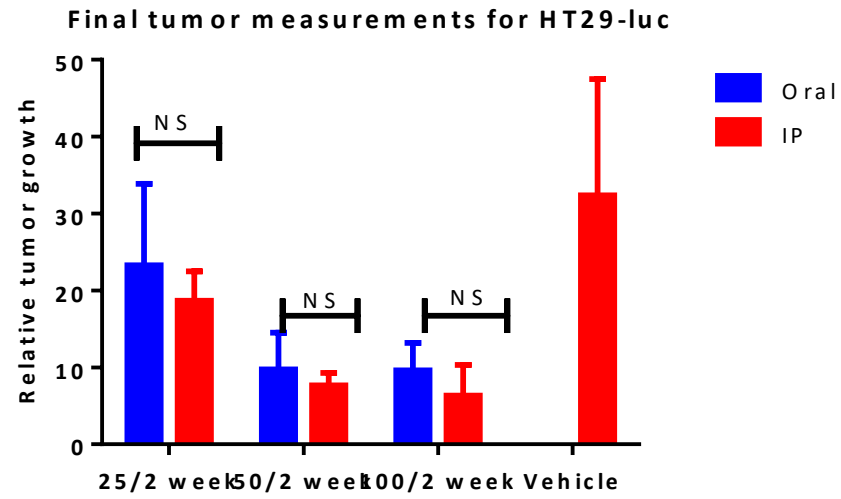

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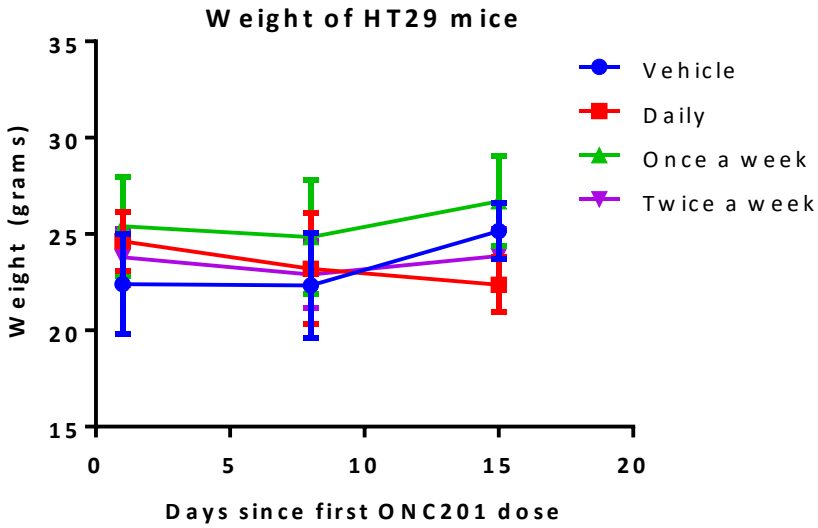

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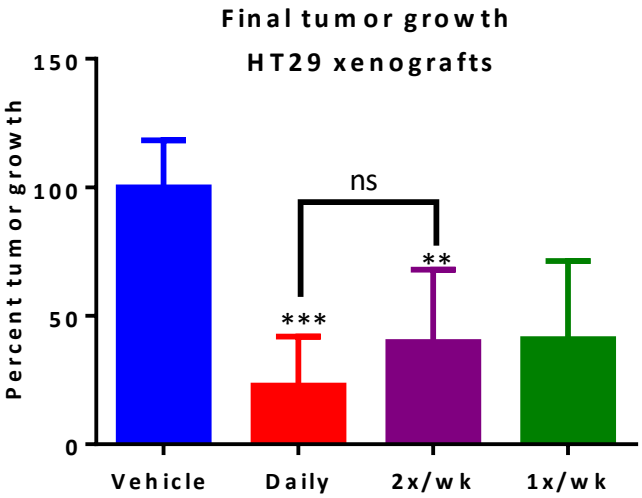

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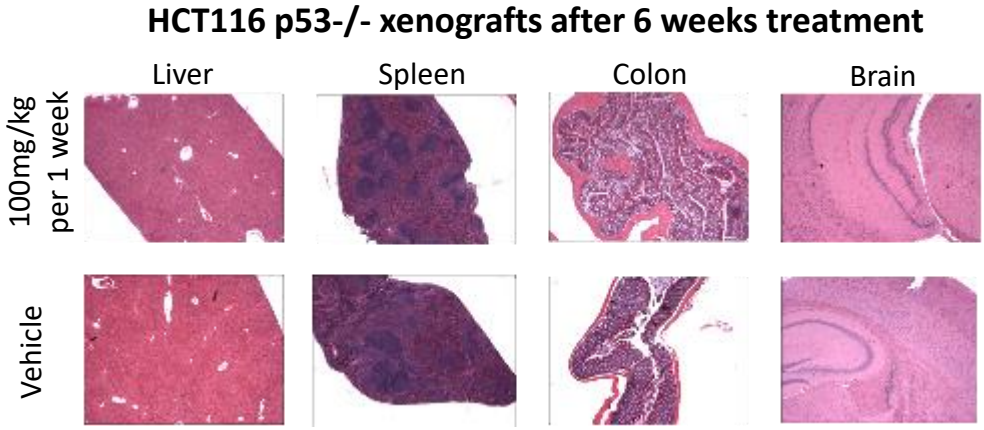
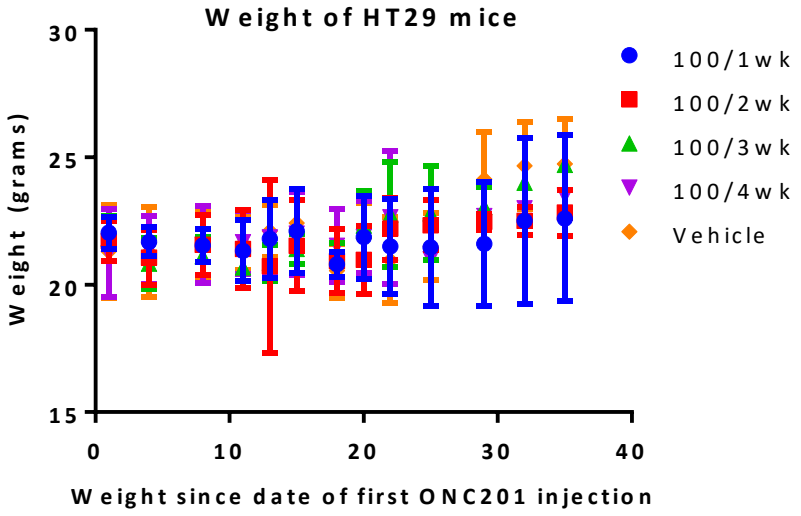


B

	Creatine	Glucose	Na+	Cl-	ALP	ALT	AST	total bilirubin	Albumin
25/1	1.57 +/- .6	212 +/- 4	107 +/- 13	116 +/- 4	38 +/- 7	120 +/- 5.6	286 +/- 3	2.8 +/- 3	2.6 +/- .15
25/2	1.4 +/- .78	286 +/- 25.1	129 +/- 13	115 +/- 4	41 +/- 12	88 +/- 25	277 +/- 15	.4 +/- .2	2.6 +/- .1
50/1	.25 +/- .05	166 +/- 6	123 +/- 8	112.5 +/-.5	38.5 +/- 10	54 +/- 15	210.5 +/- 4.5	.3 +/- .05	2.35 +/- .05
50/2	.35 +/- .05	199.5 +/- 24	108.5 +/- 12.5	88 +/-29	51 +/- 4	98 +/- 6	334.5 +/- 22.5	.2 +/- .2	2.65 +/- .15
100/1	1.45 +/- .25	205 +/- 33	129.5 +/- 18.5	116.5 +/- 3.5	41.2 +/- 1.25	88 +/- 3	217 +/- 17	.4 +/- .1	2.65 +/- .25
100/2	1.6 +/- .4	144 +/- 28	141.5 +/- 6.5	115 +/- 1	66.6 +/- 25.4	53 +/- 18	280.5 +/- 46.5	.6 +/- .2	2.7 +/- .3
vehicle	0.67 +/- .46	225 +/- 68	125 +/- 11	110 +/- 4	48 +/- 9	97.5 +/- 25.5	236 +/- 54	.47 +/- .3	2.6 +/- .08
normal range	.2-.9	125-222	140-160	110-118	75-111	17-77	54-298	0-.9	2.5-3.2

SLIGHT HEMOLYSIS
slight below
slight above
Within normal range

C



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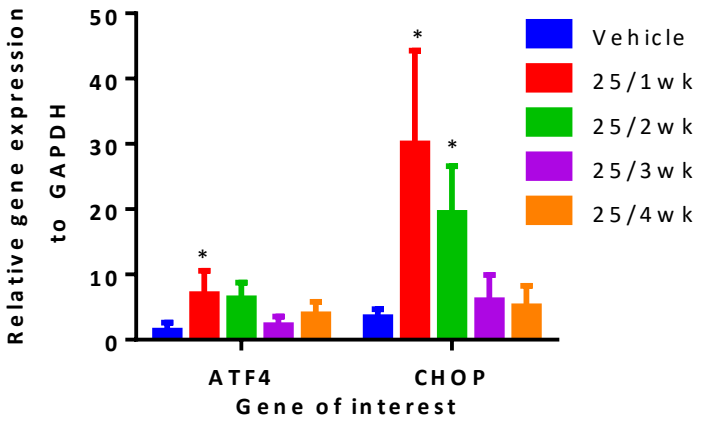
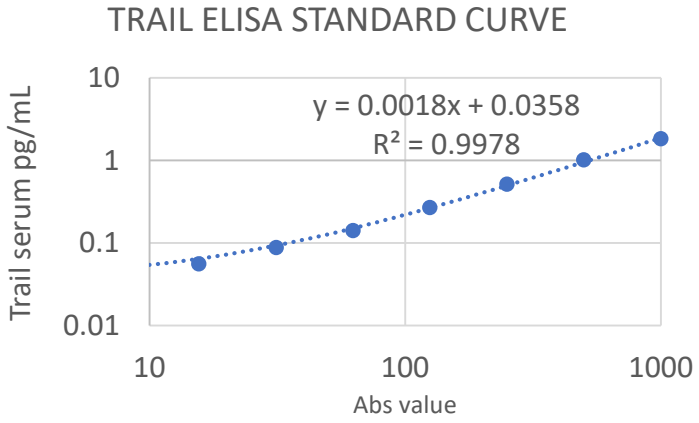


B

	Abs Value	Conc (pg/mL)
Positve Serum	0.195	91.2
Negative Serum	0.0739	21.6
Blank	0.009	0.08



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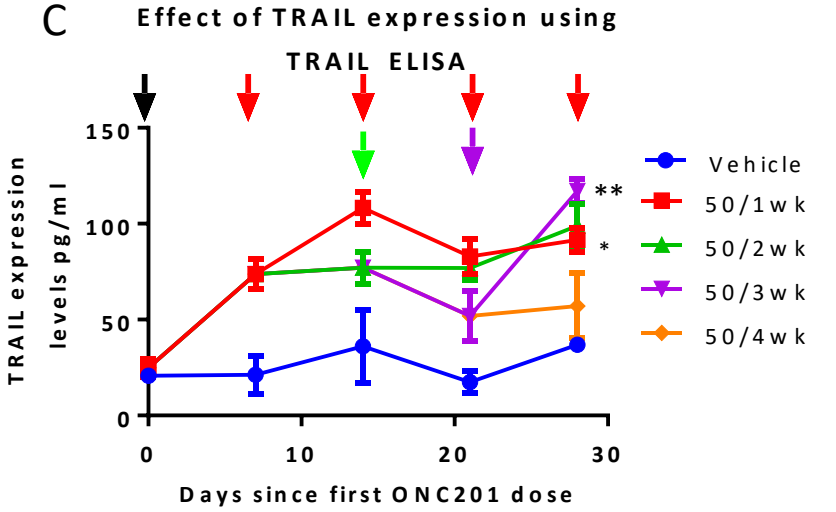

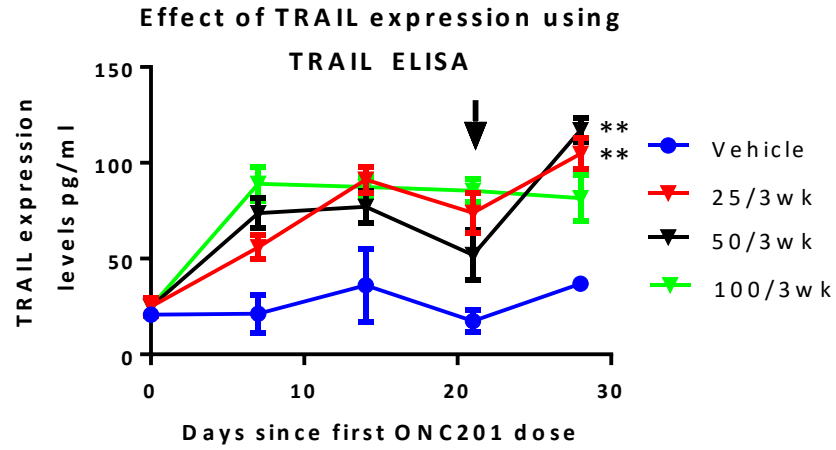

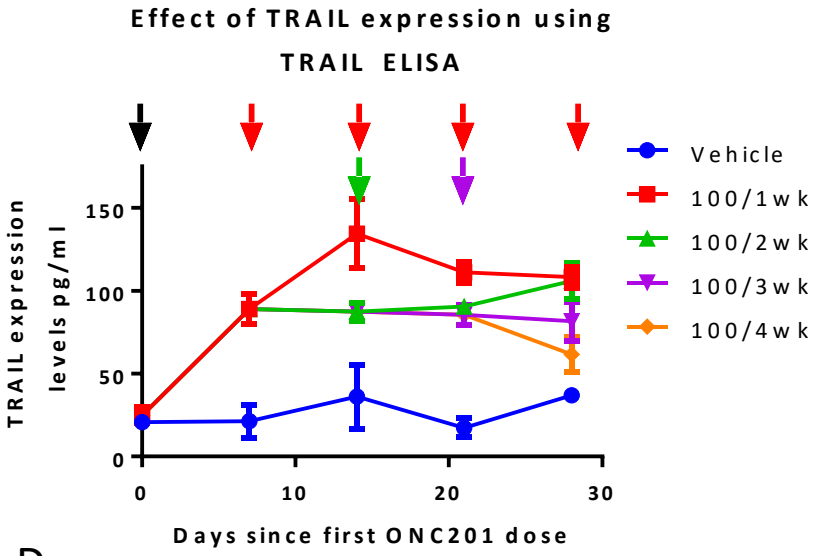

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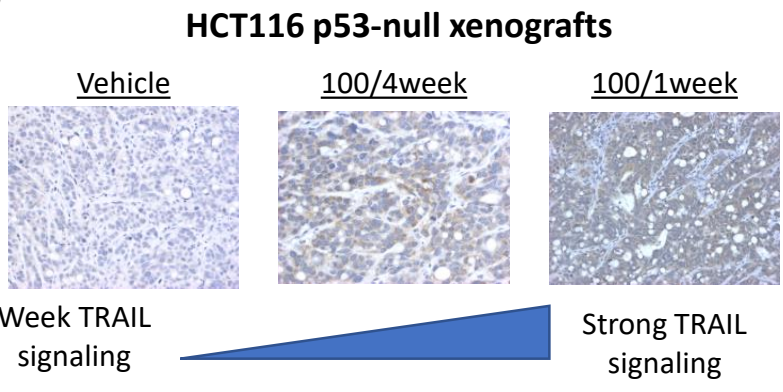

Wagner et al., Figure S4

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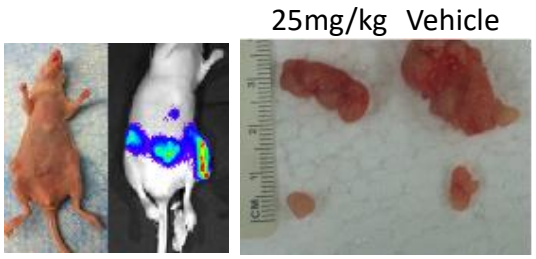

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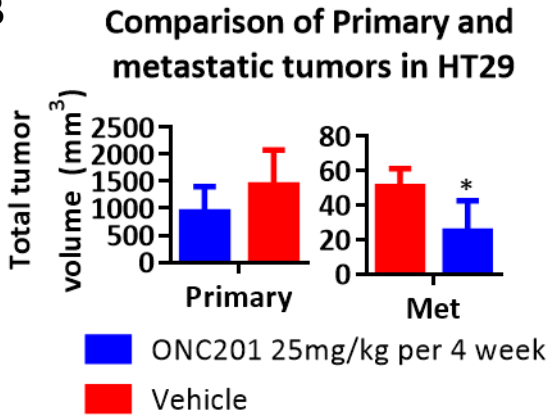

C

Cohort	Subcutaneous location: Number	Subcutaneous location: Size Range (mm^3)	Internal location (pathology)
Vehicle	8	18-40.5	liver-bt,lung-sp
25/1week	6	lung-sp	liver-sp
25/2week	2	sp-18	liver-sp
25/3week	1	sp-18	liver/lung-sp
25/4week	5	18-32	liver/lung-sp
50/1week	0		
50/2week	0		lung-sp
50/3week	0		lung-sp
50/4week	1	13.9	
100/1week	0		
100/2week	0		
100/3week	0		
100/4week	1	11.5	

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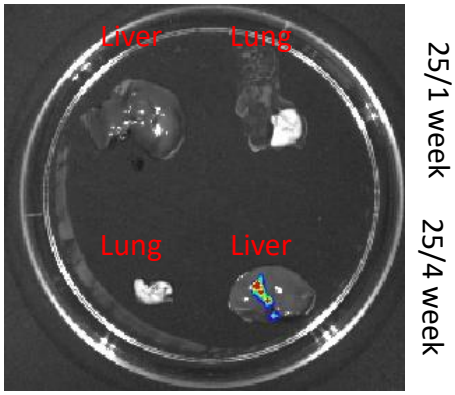

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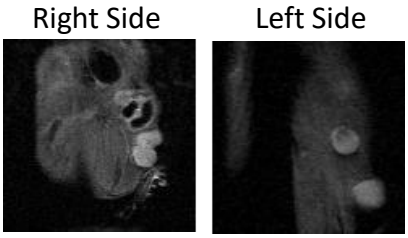

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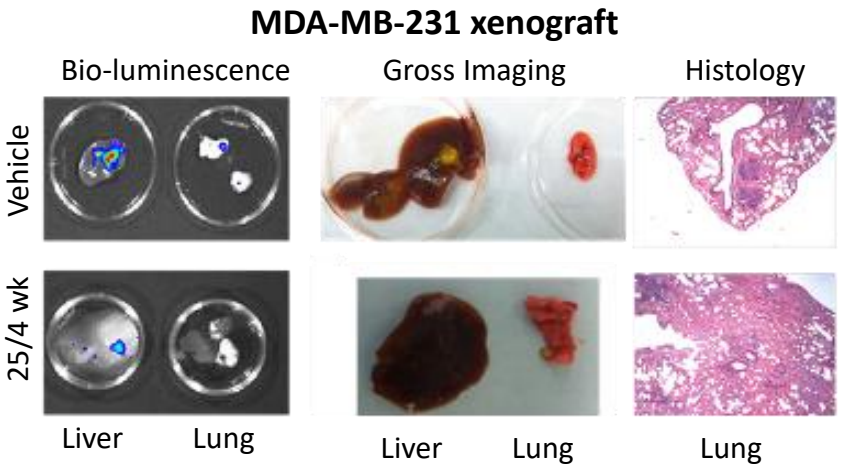

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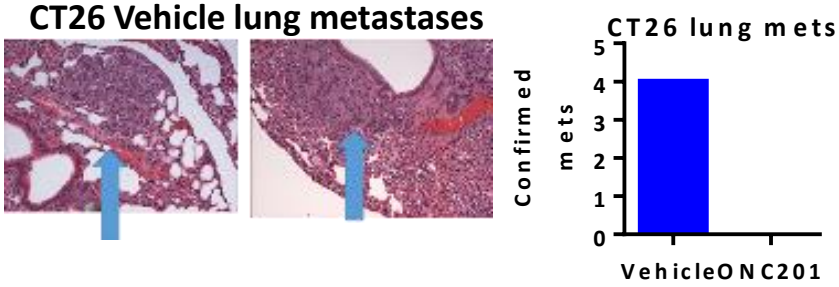

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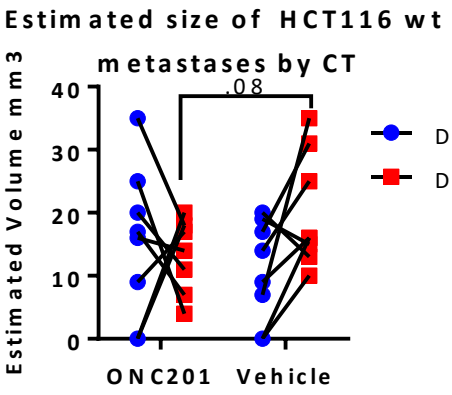

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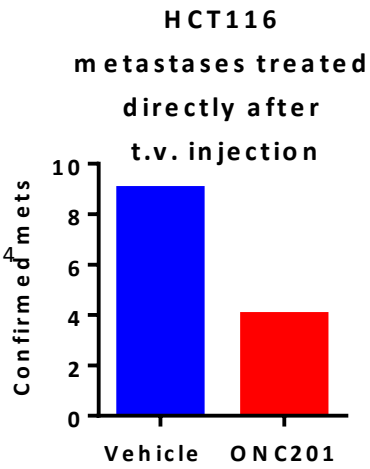
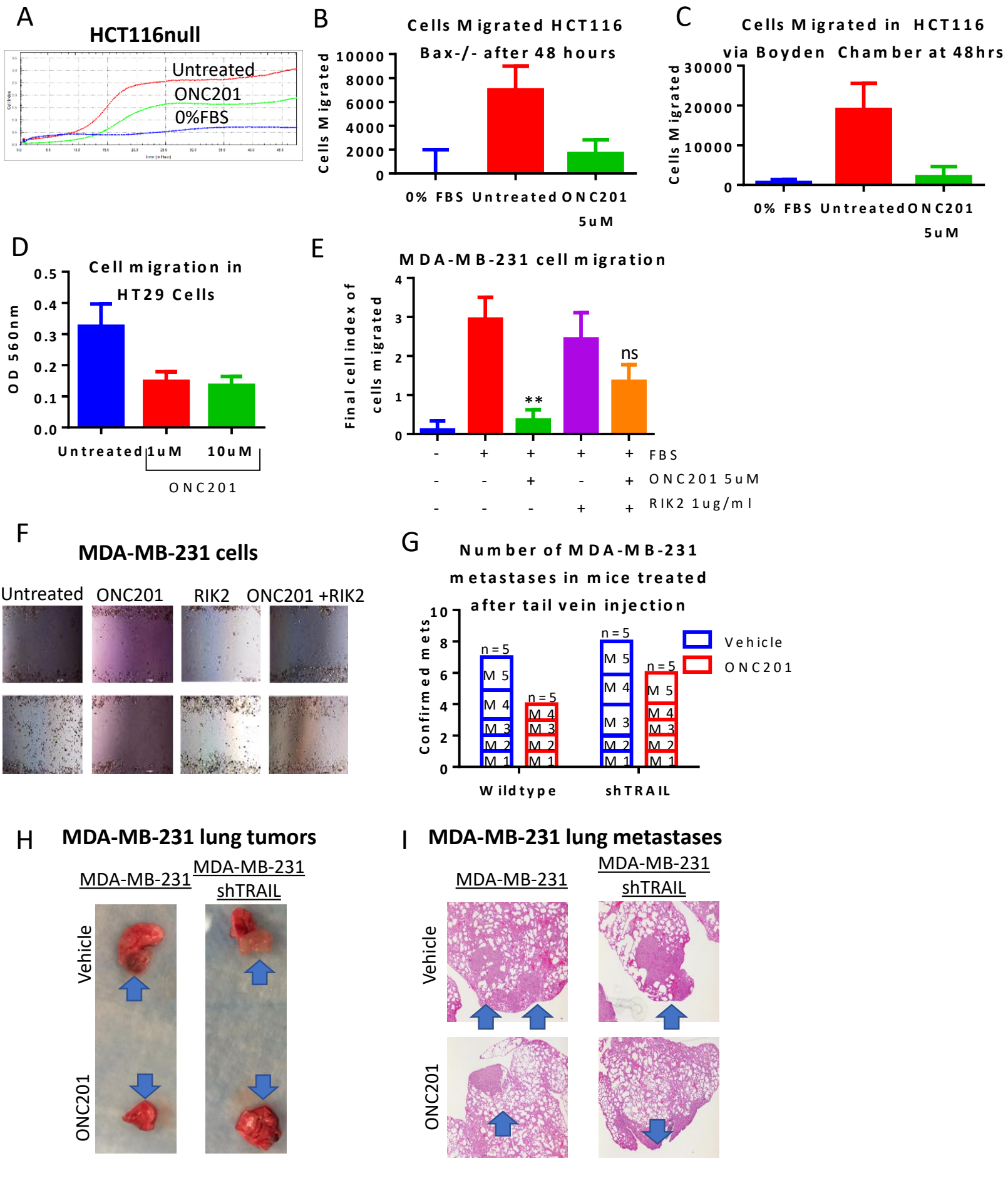


Wagner et al., Figure S5



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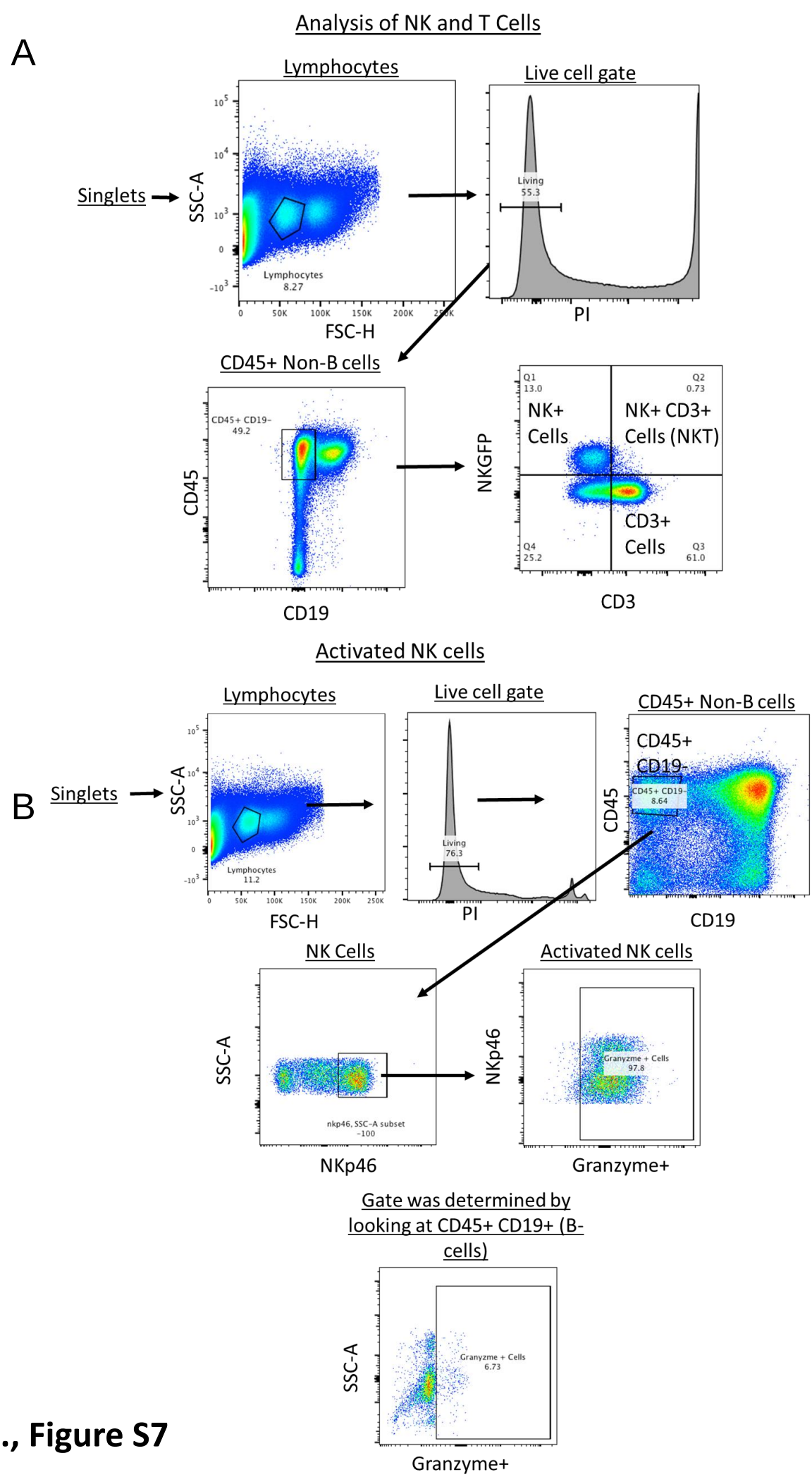

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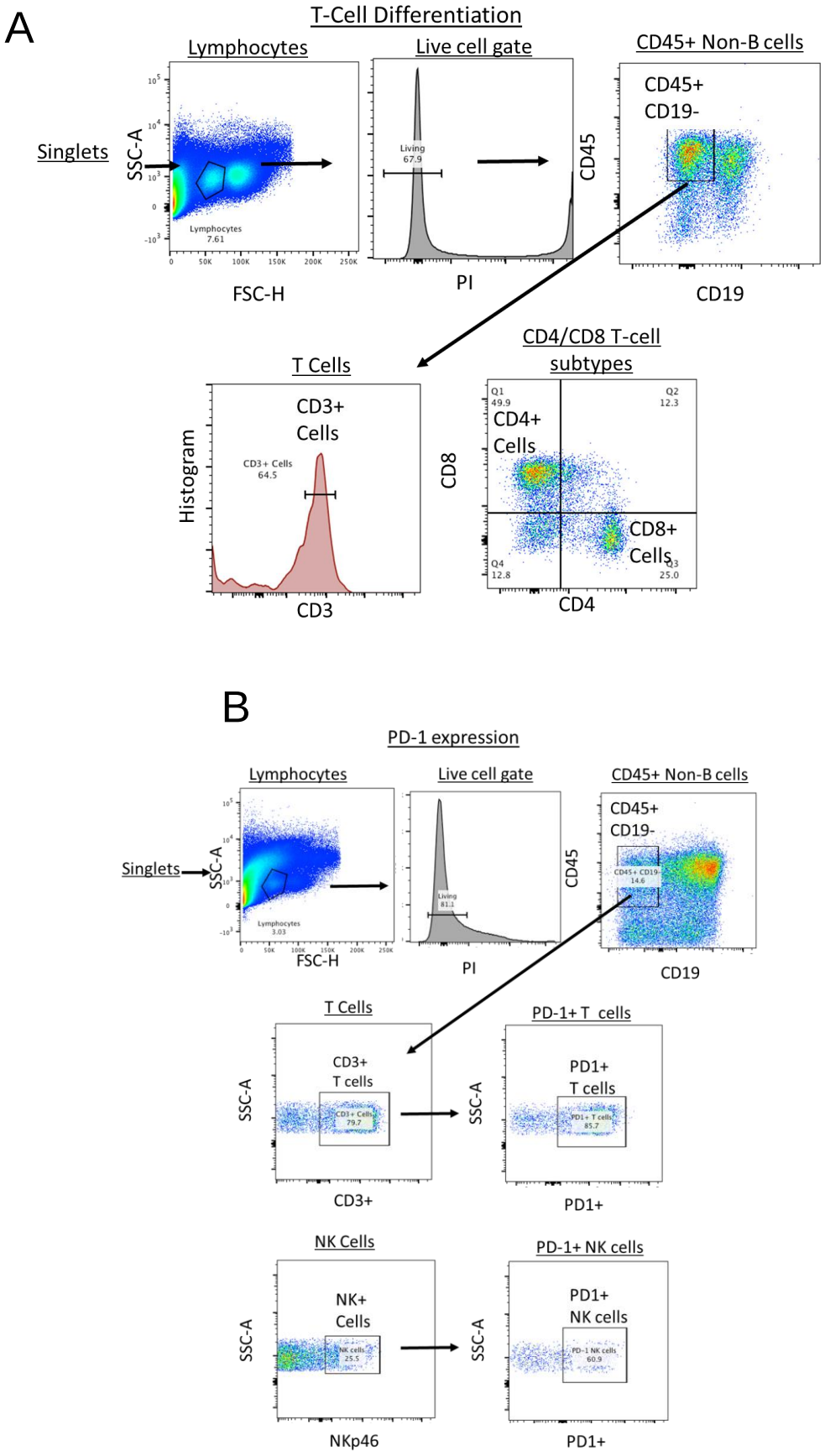

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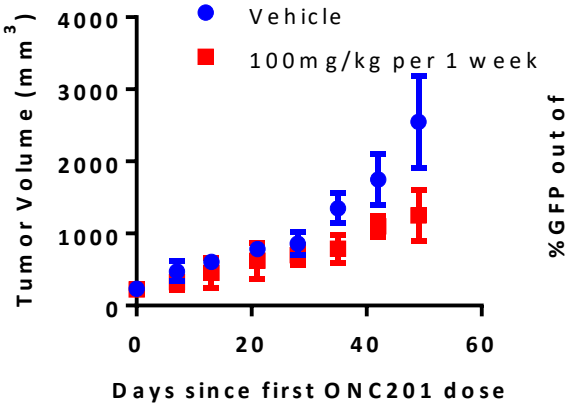

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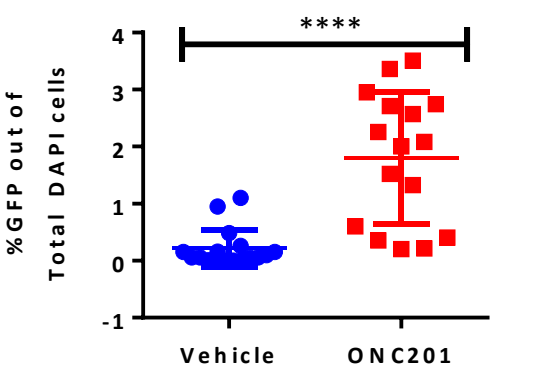

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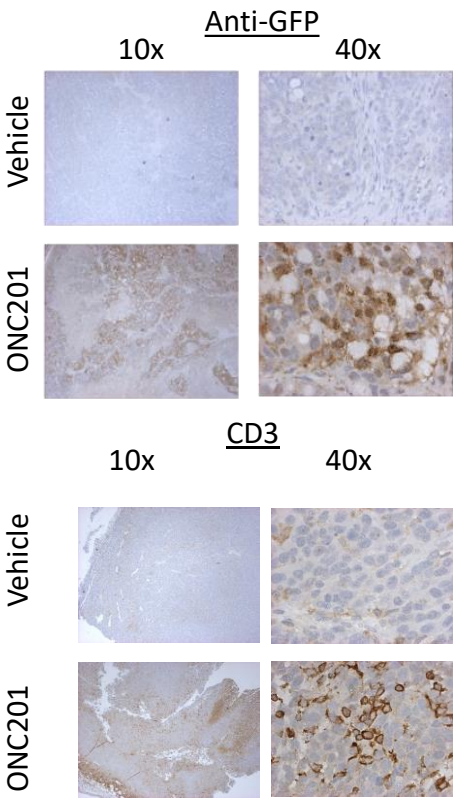
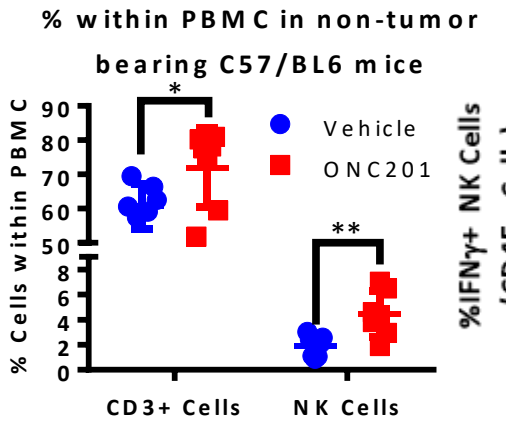


C

MC38 Xenografts

		Vehicle	ONC201	P value
% within tumor	% Lymphocytes	.86 +/- 0.52	12.58 +/- 6.97	0.036
	%CDllb+/Ly6C+	0.002 +/- .003	.095+/- .005	ns
	%CDllb/Ly6G+	.014+/- .02	.231 +/- .032	0.03
% within lymph population				
	%NK Cells	1.94 +/- 1.99	10.99 +/- 7.95	0.055
	% CD3+ Cells	7.26 +/- 10.18	40.5 +/- 8.85	0.005
% within CD3+ cells				
	CD4+/CD3+ Cells	7.31 +/- 5.16	54.28 +/- 7.48	0.004
	CD8+/CD3+ Cells	5.56 +/- 3.88	35.68 +/- 5.99	0.004

E



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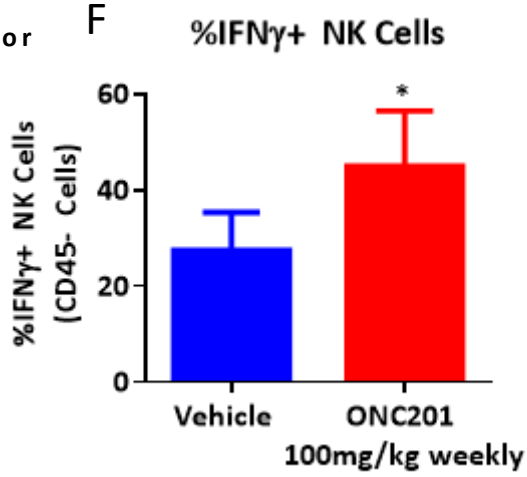

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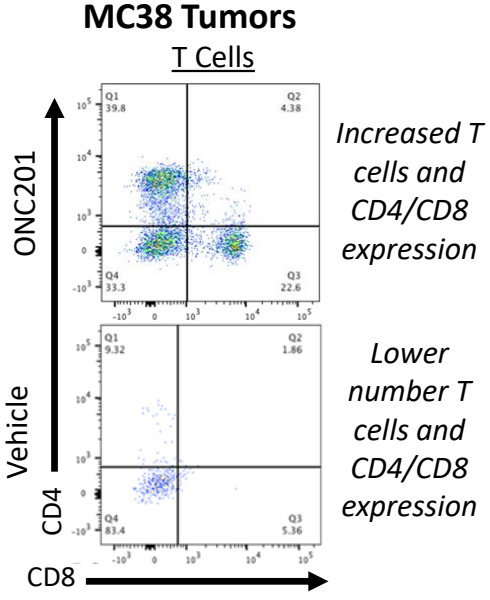
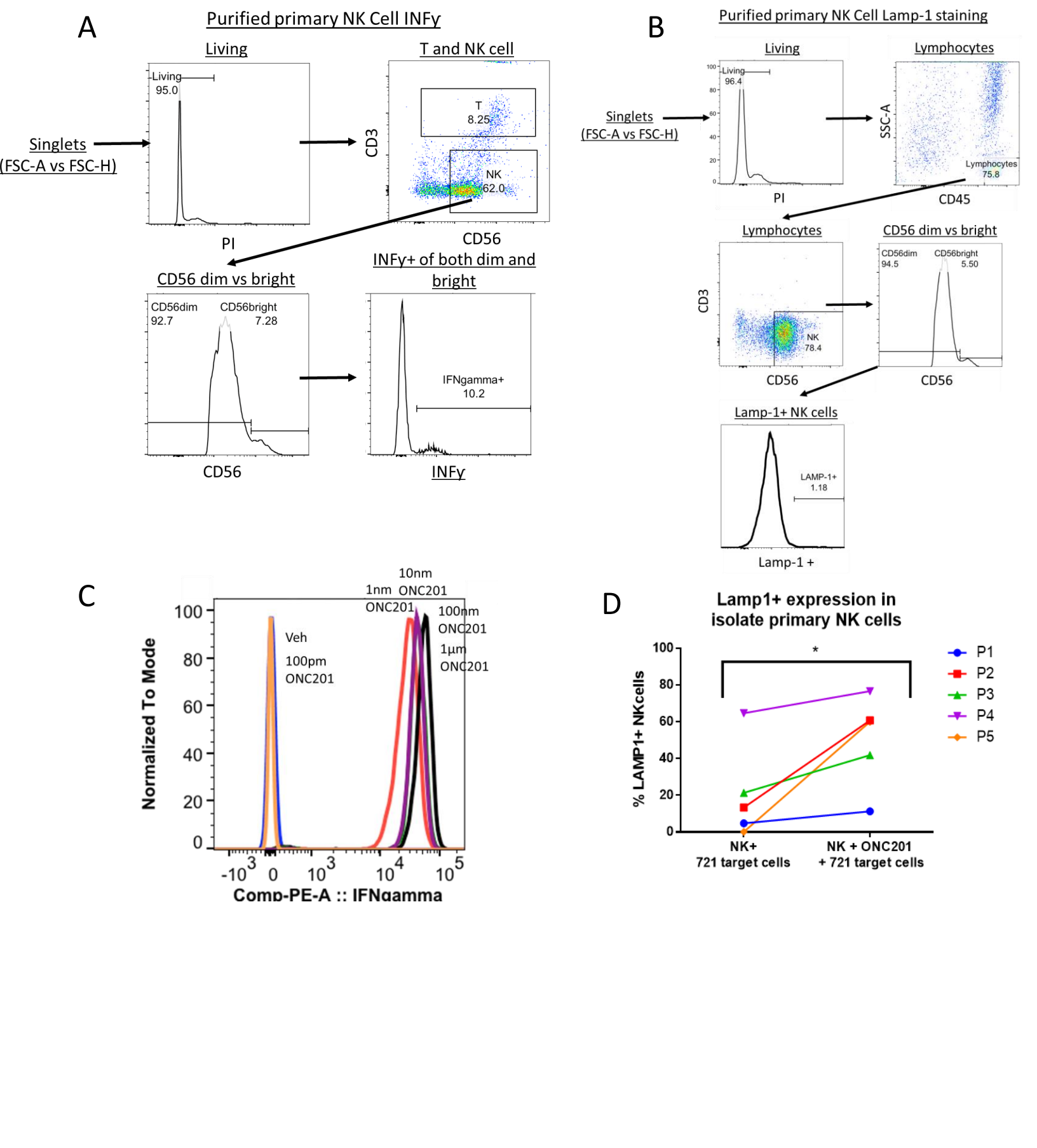


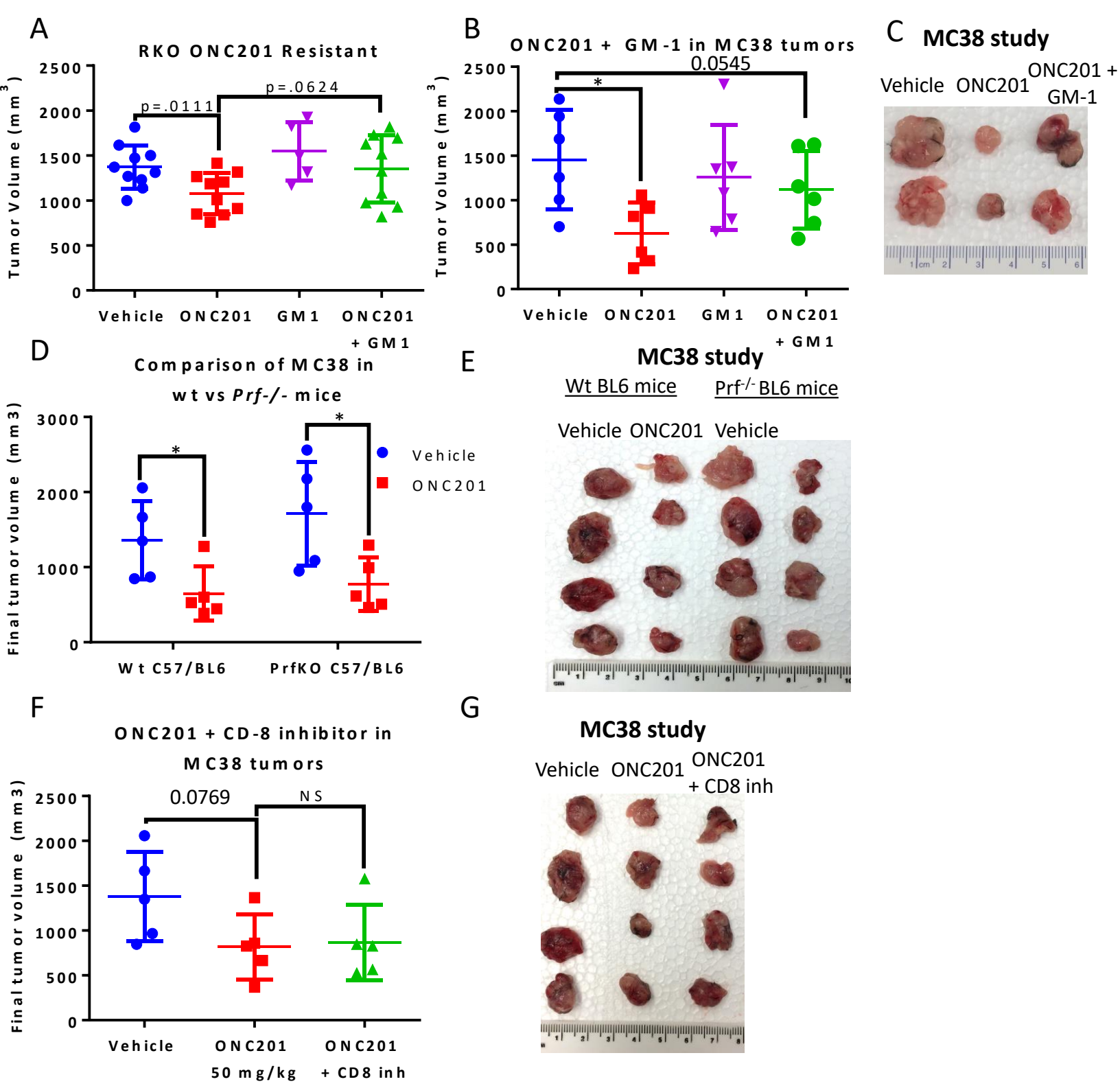
Increased T cells and CD4/CD8 expression

Lower number T cells and CD4/CD8 expression

Wagner et al., Figure S9



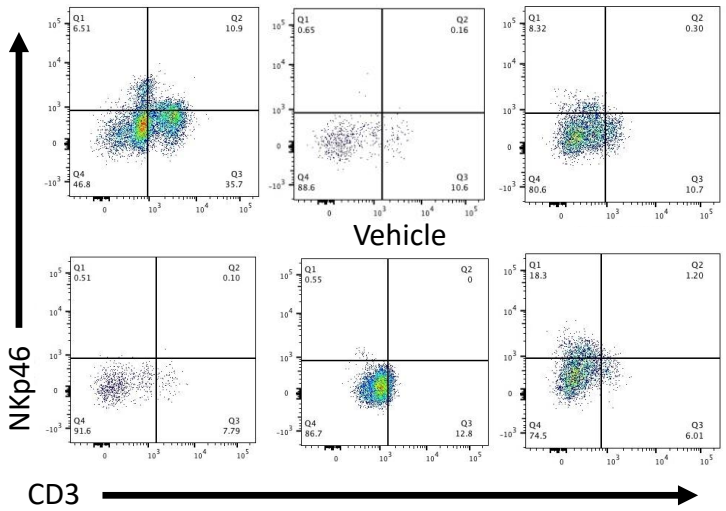
Wagner et al., Figure S10



Wagner et al., Figure S11

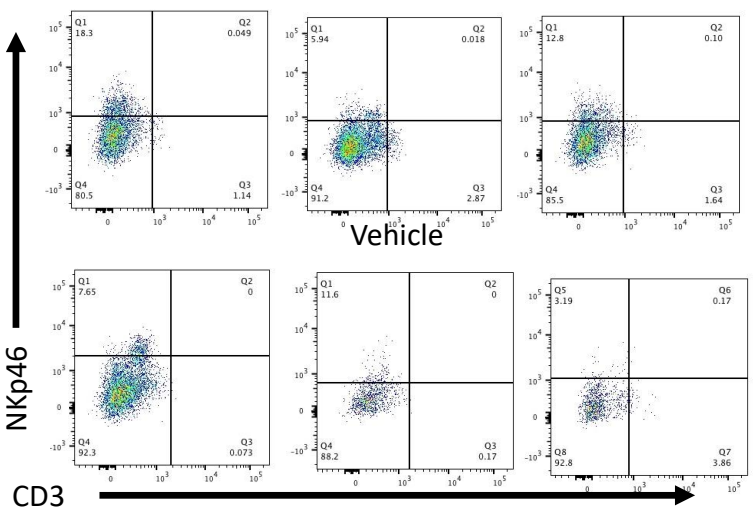
A

HCT116 lung metastases (athymic nude mice)



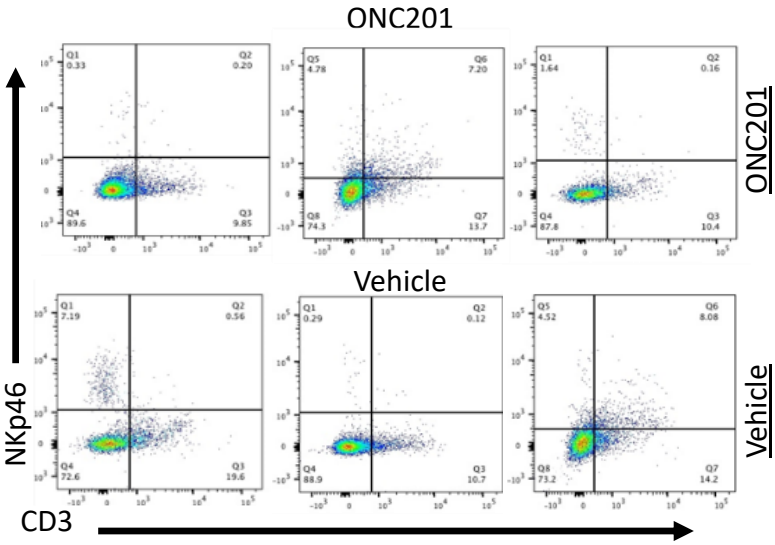
B

HCT116 Bax-/- lung metastases (athymic nude mice)



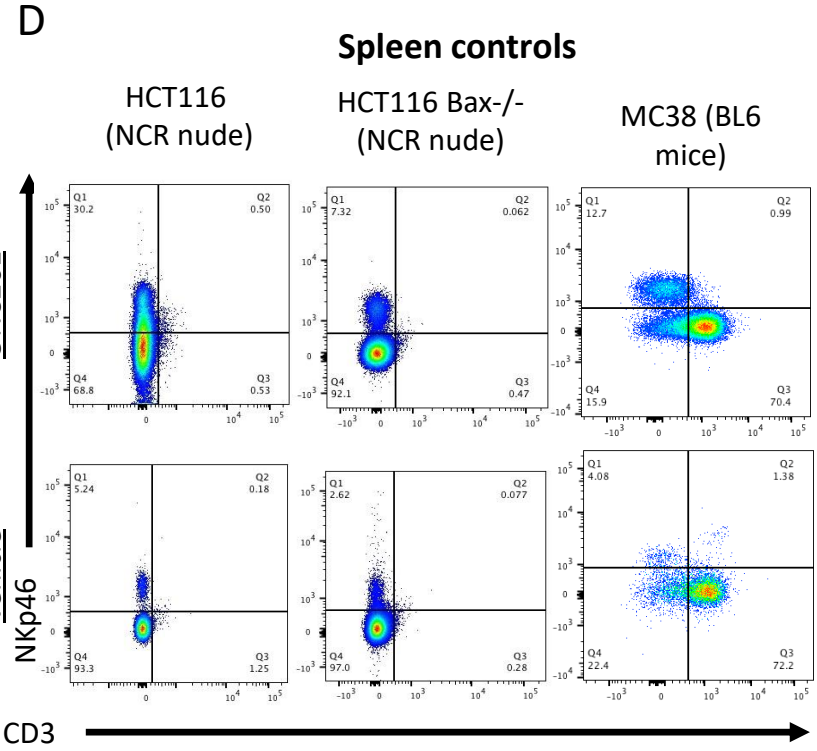
C

MC38 lung metastases (C57/BL6 mice)

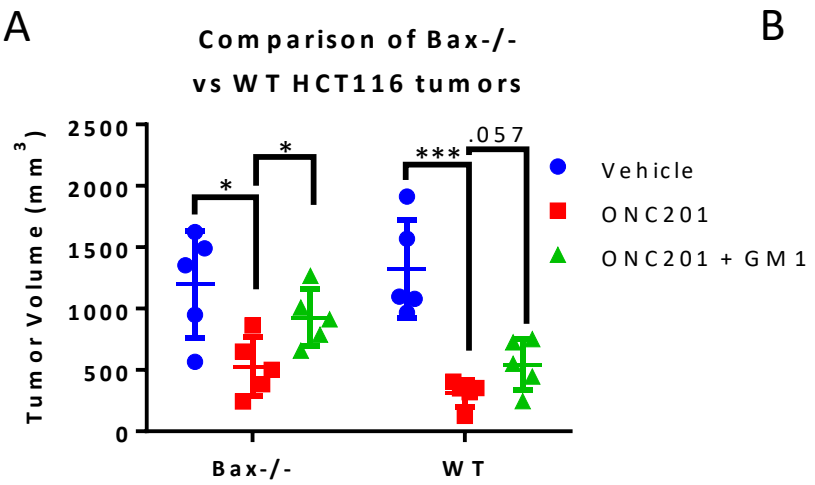


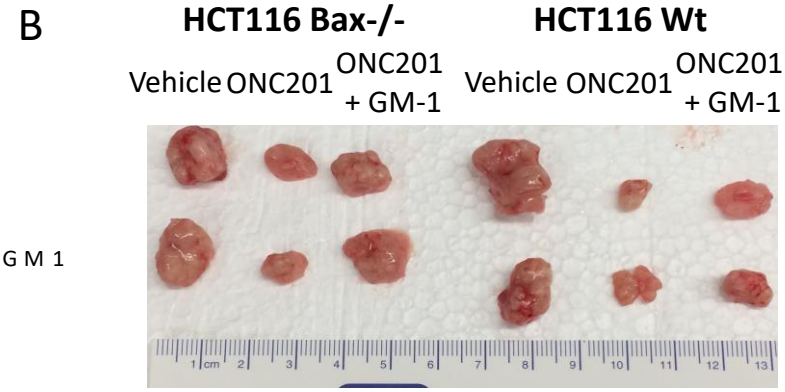
D

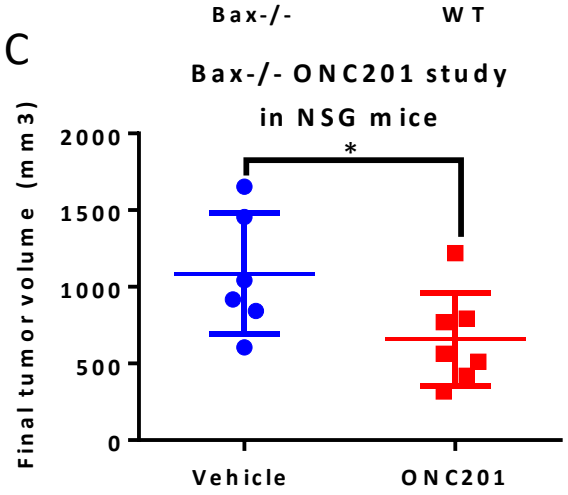
Spleen controls

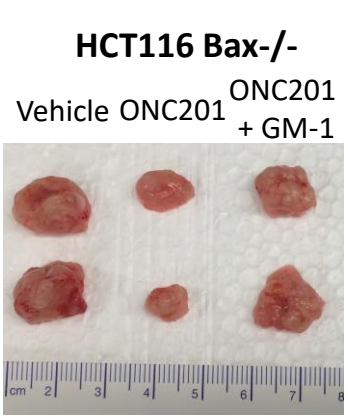


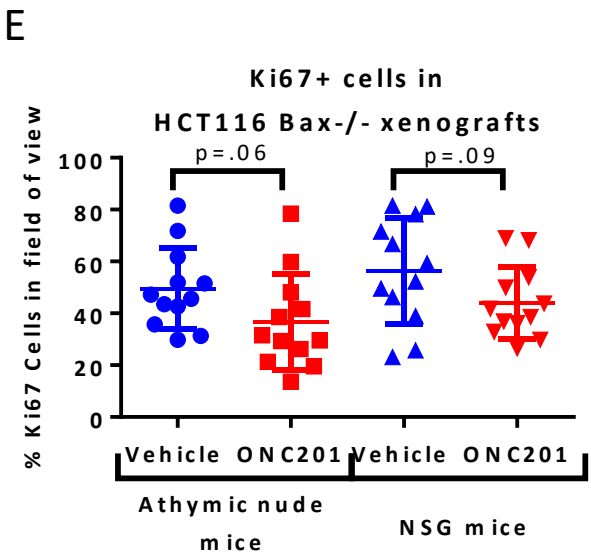
Wagner et al., Figure S12

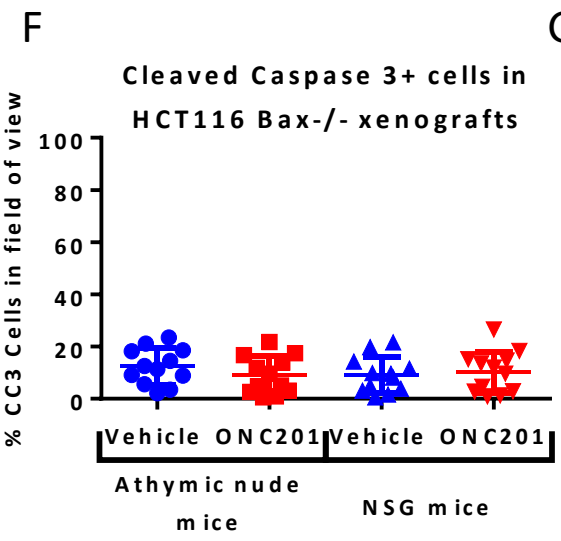


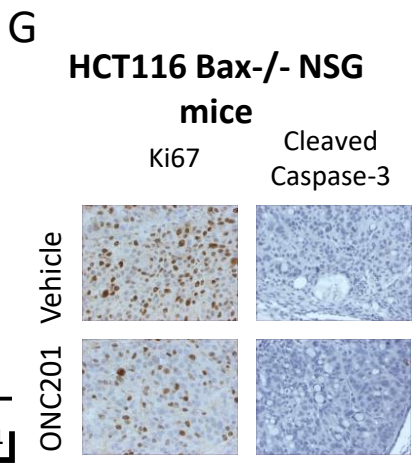




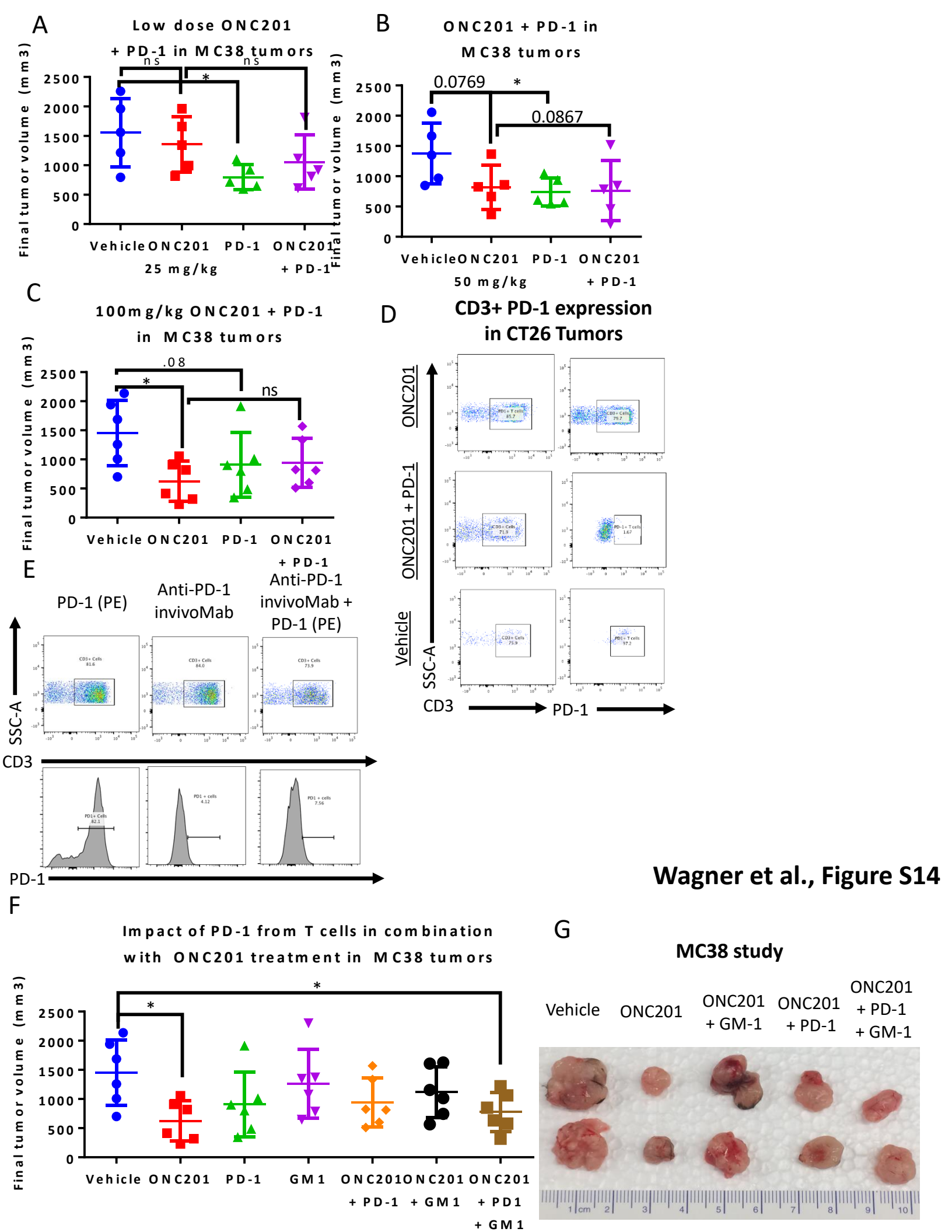






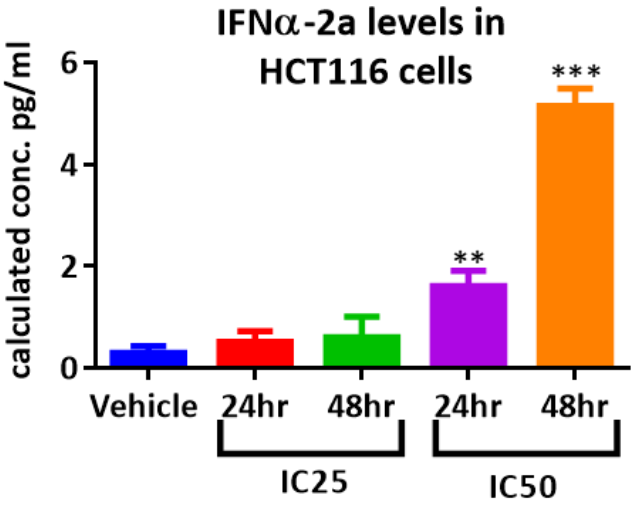


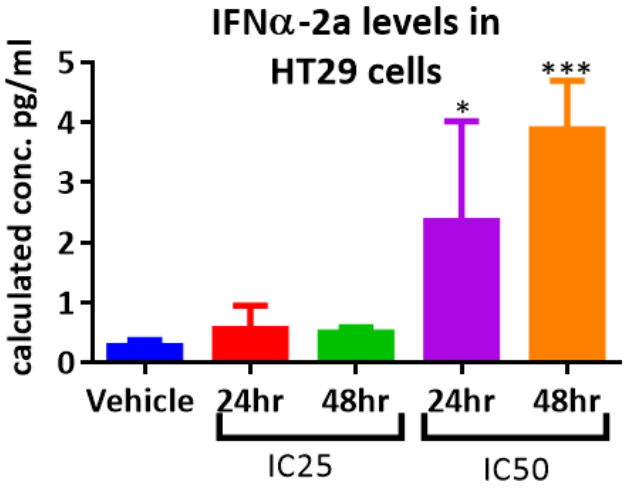
Wagner et al., Figure S13

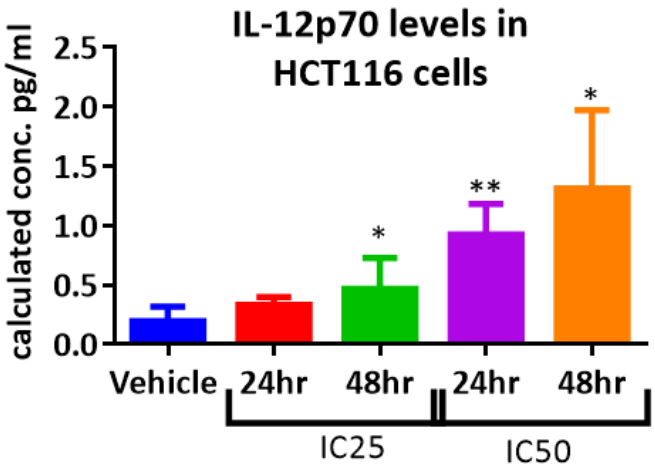


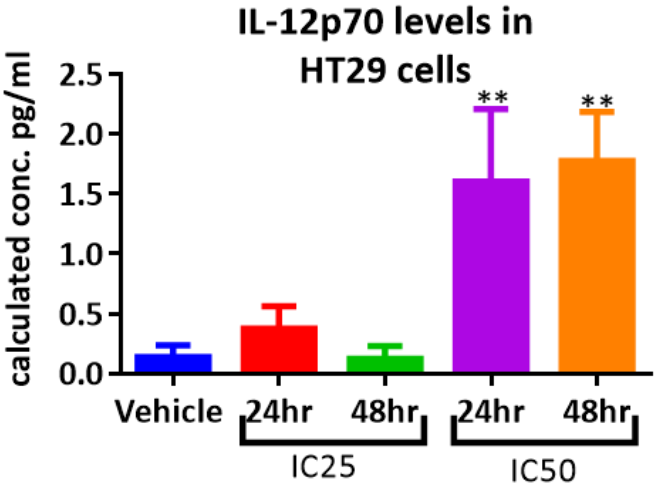
Wagner et al., Figure S14

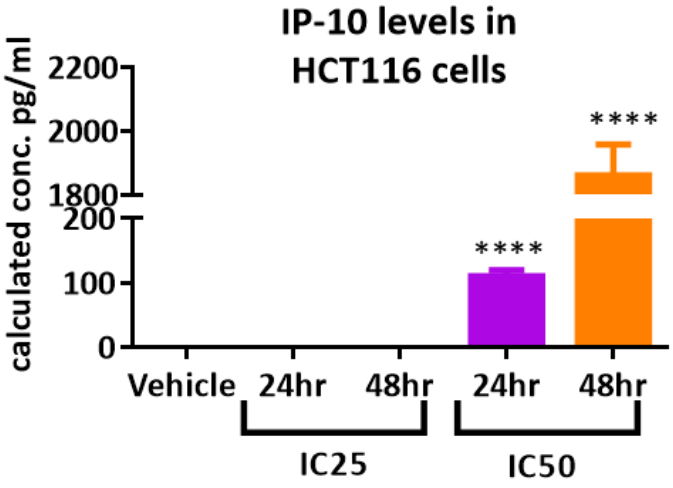
Tumor conditioned media results from ELISA Multiplex

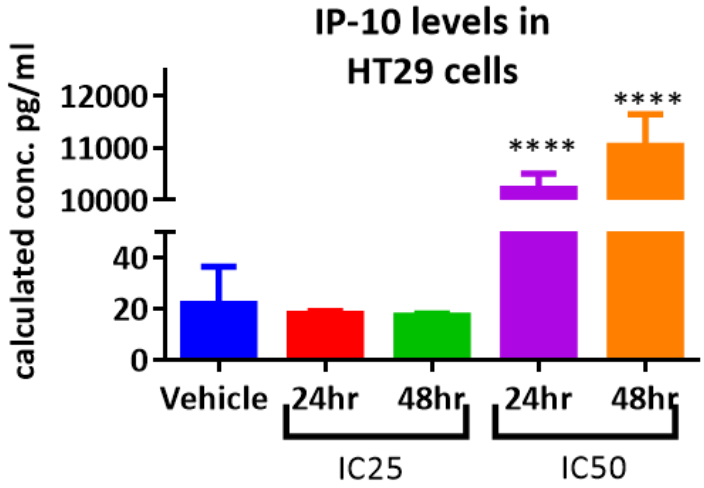


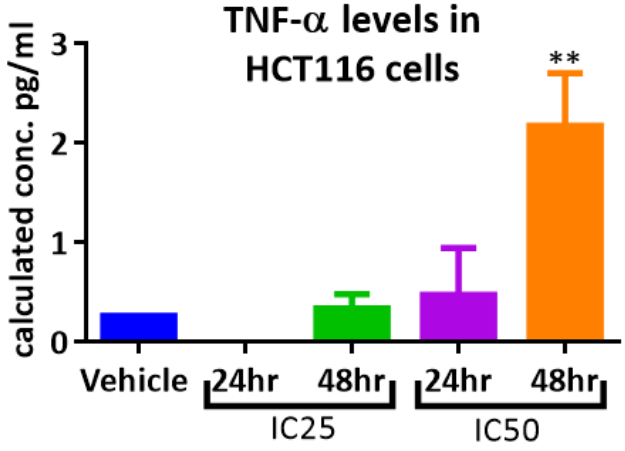


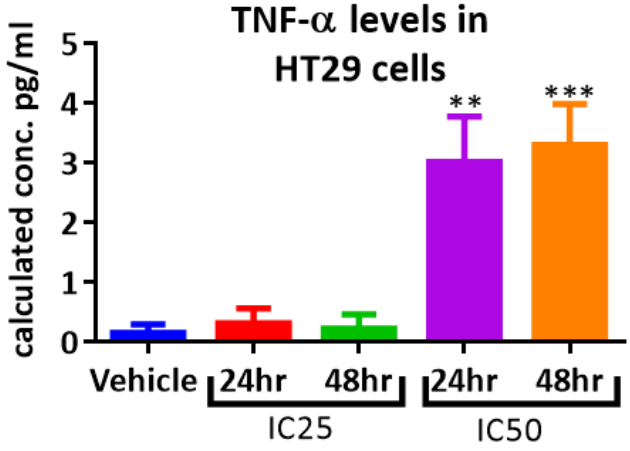






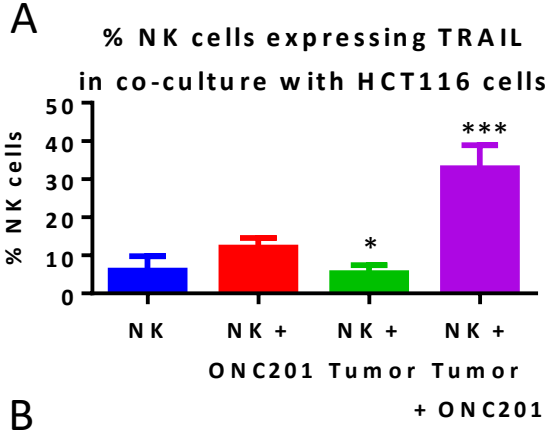


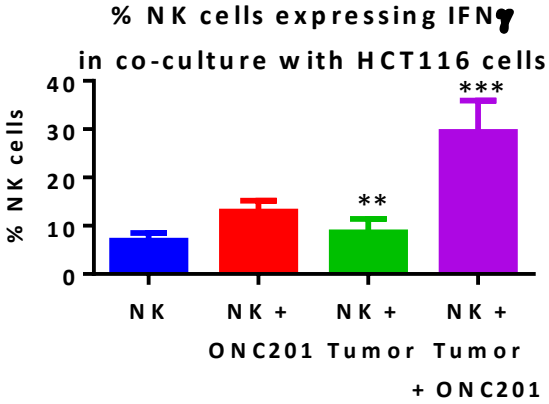




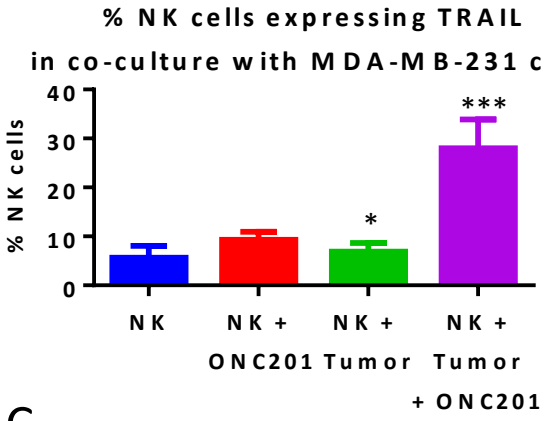
Wagner et al., Figure S15

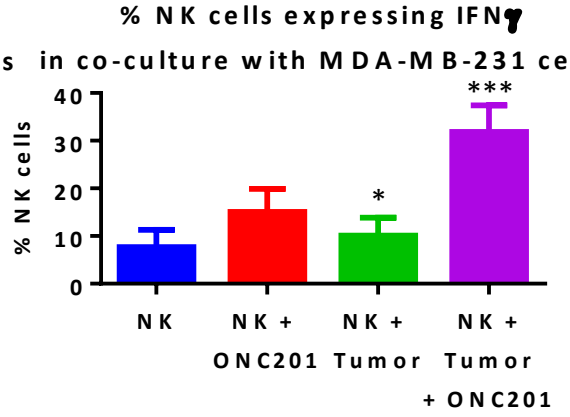
A



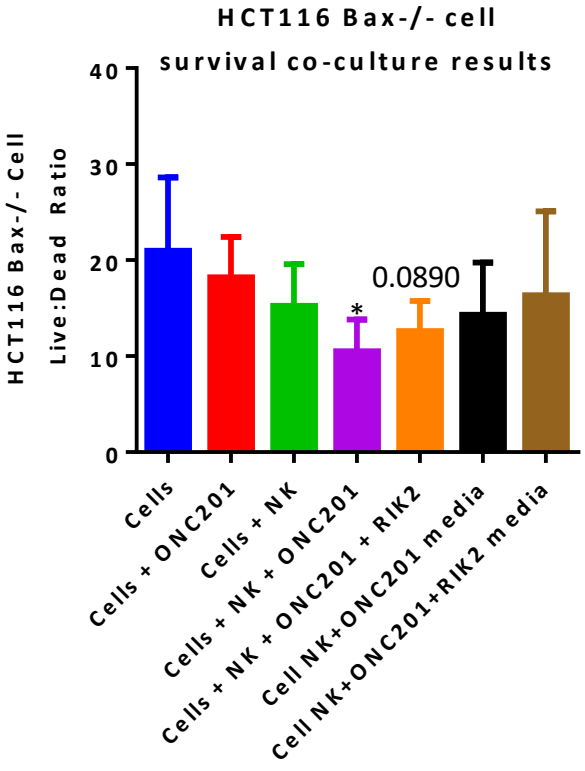


B



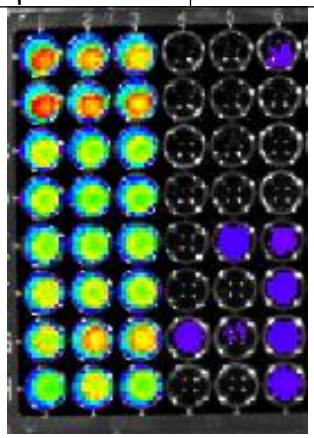


C



D

	Viability	St. Dev.
Cells	100.00%	8.31%
Cells + ONC201	83.90%	2.59%
Cells + ONC201 + RIK2	89.14%	3.53%
Cells + NK	91.94%	10.24%
Cells + NK + ONC201	2.08%	1.36%
Cells + NK + ONC201 + RIK2	1.47%	0.46%
Cells + NK + IL-2	4.00%	3.39%
Cells + NK + IFNγ	4.41%	3.23%



E

Cells Untreated	Cells + NK pretreated with ONC201 10uM
Cells + ONC201 + 10uM	Cells + NK pretreated with ONC201 10uM + RIK2 2ug/ml
Cells + ONC201 + 10uM + RIK2 2ug/ml	Cells + NK pretreated with IL-2
Cells + NK alone	Cells + NK pretreated with IFNγ

Wagner et al., Figure S16

Supplemental Table 1: Antibody Chart

	Reactivity	Company	Catalogue Number
pERK	Human/Mouse	CS	9106s
p-Akt	Human/Mouse	CS	9275s
ERK	Human/Mouse	CS	9102s
Akt	Human/Mouse	CS	4685s
β-actin	Human/Mouse	Sigma	A5441
NKp46 mouse (Flow)	Mouse	eBioscience	48-3351-82/11-3351-82
CD3 (Flow)	Mouse	eBioscience	47-0032-80
CD45 (Flow)	Mouse	eBioscience	83-0451-41
CD19 (Flow)	Mouse	eBioscience	17-0193-80/12-0193-82
EPCAM-1 human (Flow)	Human	eBioscience	53-8326-41
EPCAM-1 mouse (flow)	Mouse	eBioscience	11-5791-80
IFNγ (Flow)	Mouse	eBioscience	11-7311-41
Granzyme B (Flow)	Mouse	eBioscience	11-8898-80
GM-1	Mouse	Wako	986-10001
TRAIL (IHC)	Human	CS	3219S
NK1.1 (Flow)	Mouse	eBioscience	12-5941-81
CD11b(Flow)	Mouse	Biolegend	101225
TRAIL mouse(Flow)	Mouse	Biolegend	109305
TRAIL human (Flow)	Human	Biolegend	308206
F4/80 (Flow)	Mouse	Thermo/Fisher	MF48000
Ly6G/GR-1 (Flow)	Mouse	eBioscience	127605
NKp46 (IF/IHC)	Mouse	R&D Systems	AF2225-SP
PD-1 (mouse/IHC)	Mouse	Biolegend	114101
CD27 (Flow)	Mouse	eBioscience	46-0271-80
EPCAM-1 human (IF)	Human	CS	2929S
CD56/NK human (IF)	Human	Biolegend	318309
Ki67 (IHC)	Mouse	Dako	M7249
Ki67 (IHC)	Human	Dako	M7240
Cleaved Caspase-3	Human	CS	9661
PD-1 (IF)	Mouse	eBioscience	12-9981-81
CD-8a (IF)	Mouse	eBioscience	17-0081-81
IFNγ secretion Assay (Flow)	Human	Miltenyi Biotech	130-054-202
LAMP 1+ (Flow Human Primary)	Human	Biolegend	3208608
CD56 (Flow Human Primary)	Human	BD Bioscience	563554
CD45 (Flow Human Primary)	Human	eBioscience	45-9459-42
CD3 (Flow Human Primary)	Human	BD Bioscience	560176
CD3 (Human Study)	Human	eBioscience	56-0037-42
CD19 (Human Study)	Human	Biolegend	302252
CD56 (Human Study)	Human	BD Bioscience	335791
Granzyme B (Human Study)	Human	Biolegend	515406
TRAIL (Human Study)	Human	eBioscience	12-9927-42
LIVE/DEAD (Human Study)	-------	Invitrogen	L34966