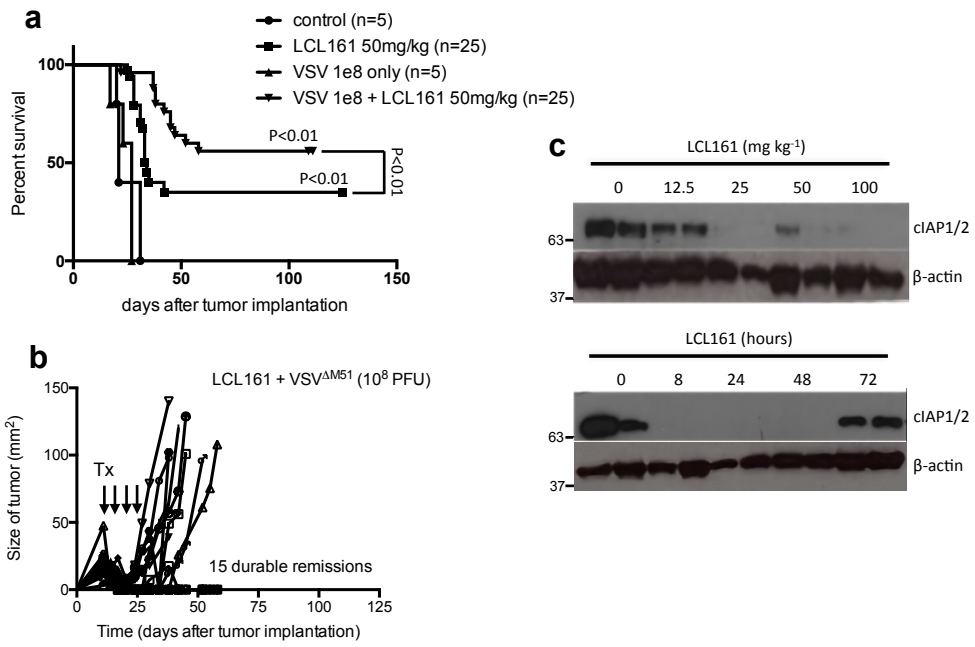
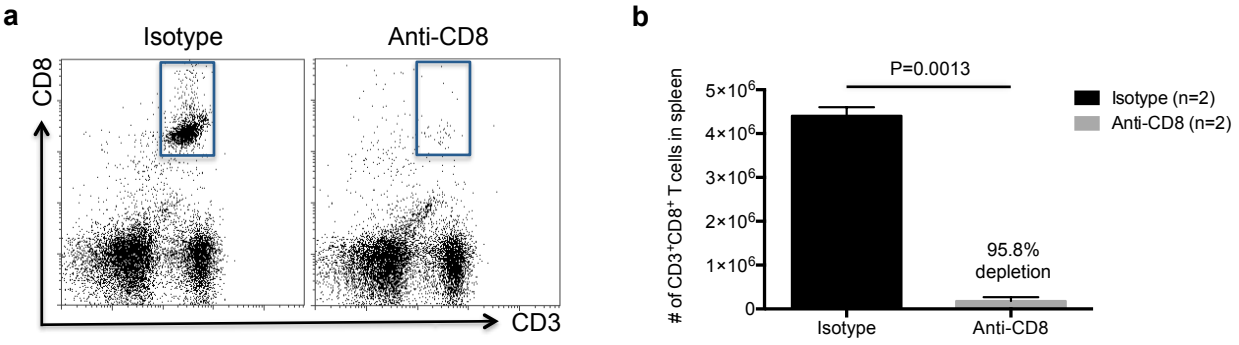
Description of Supplementary Files

File Name: Supplementary Information
Description: Supplementary Figures and Supplementary Table

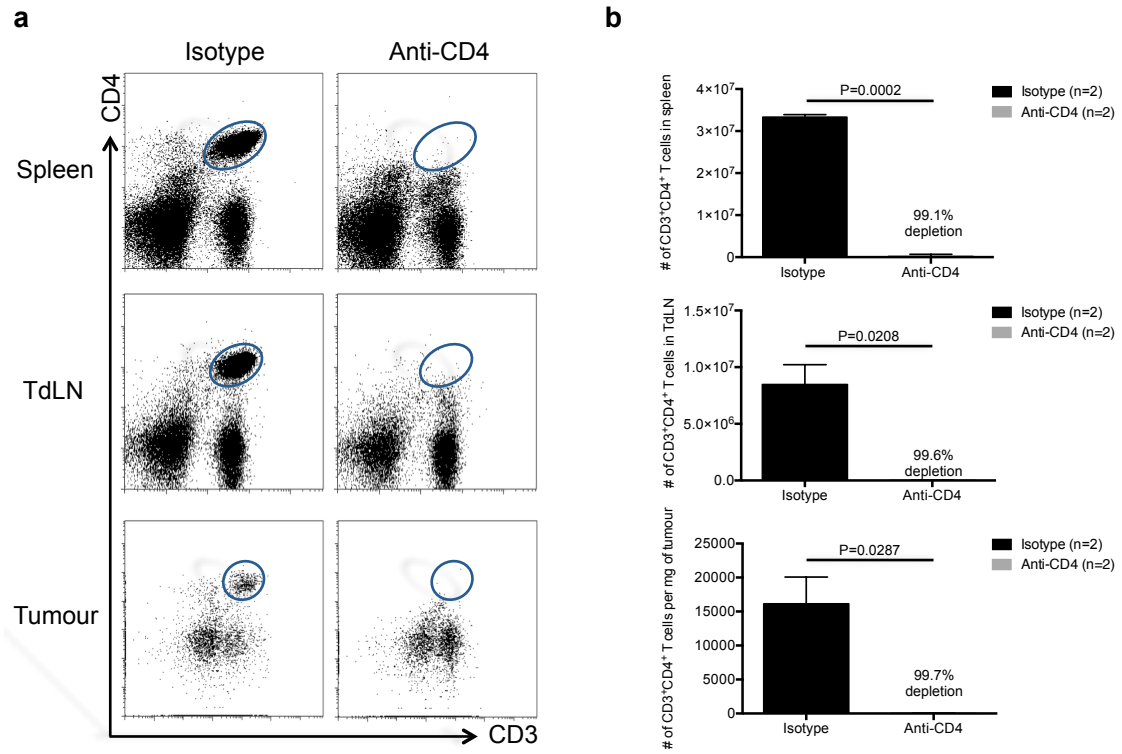
File Name: Peer Review File
Description:



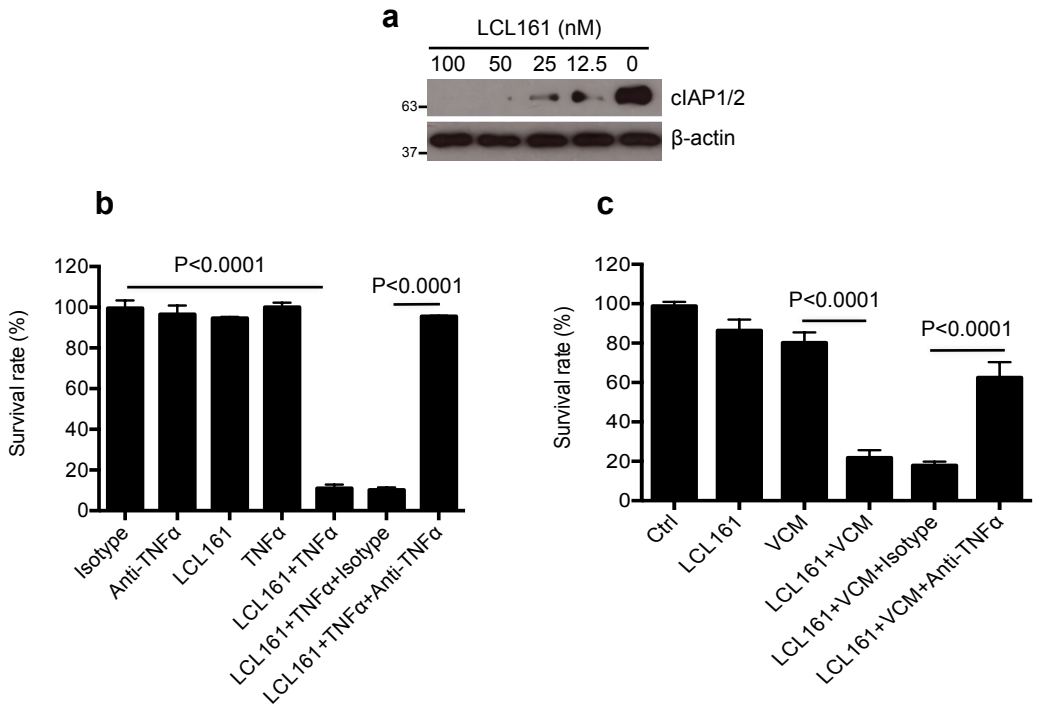
Supplementary Figure 1. LCL161±VSV$^{\Delta M51}$ therapy can elicit complete and durable EMT6 tumour control in Balb/c mice. **(a)** Overall survival of EMT6 tumour-bearing mice in response to the indicated treatments (triplicate experiments; log-rank test). **(b)** EMT6 tumour growth rate in response to LCL161+VSV$^{\Delta M51}$ combination therapy, measured by skin calipers. **(c)** cIAP1/2 protein in EMT6 tumours extracted from mice treated with LCL161, detected by immunoblots (n=2 biological replicates per experiment, duplicate experiments).



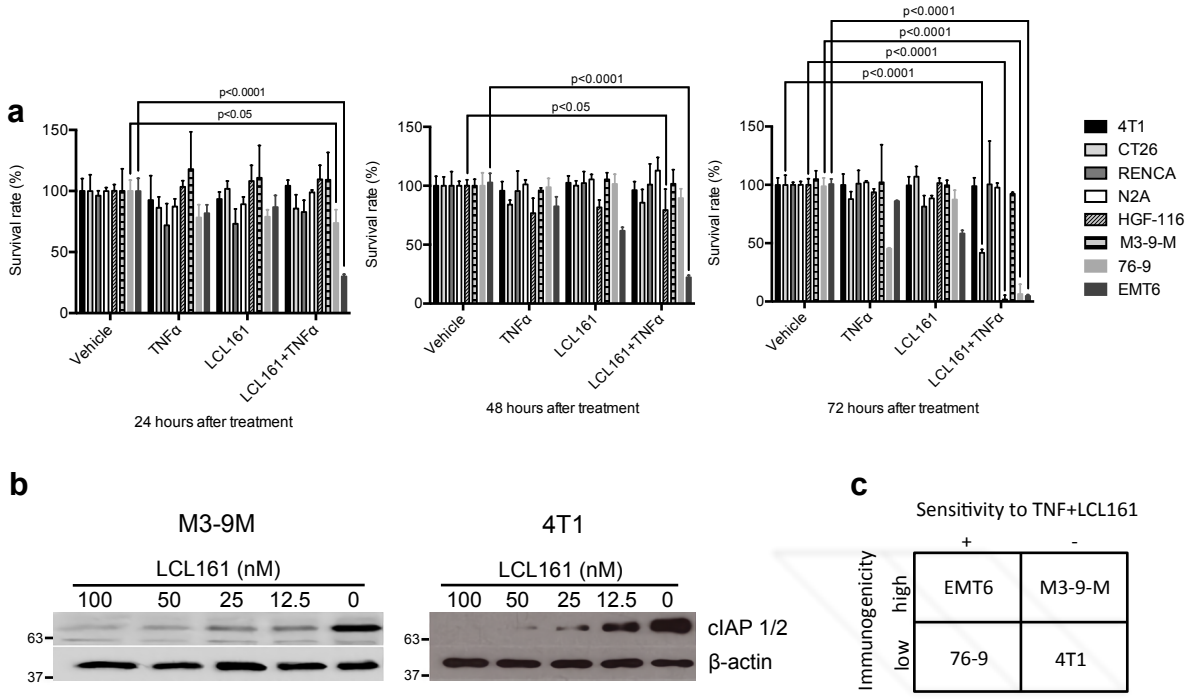
Supplementary Figure 2. Flow cytometry for $CD8^+$ T cells following neutralizing antibody treatment. **(a)** Representative flow cytometry dot plots of splenic $CD3^+/CD8^+$ T cells. **(b)** Splenic $CD3^+CD8^+$ T cells 3 days after αCD8 (or isotype) antibody treatment, measured by flow cytometry (duplicate experiments; mean±SD; *t*-test).



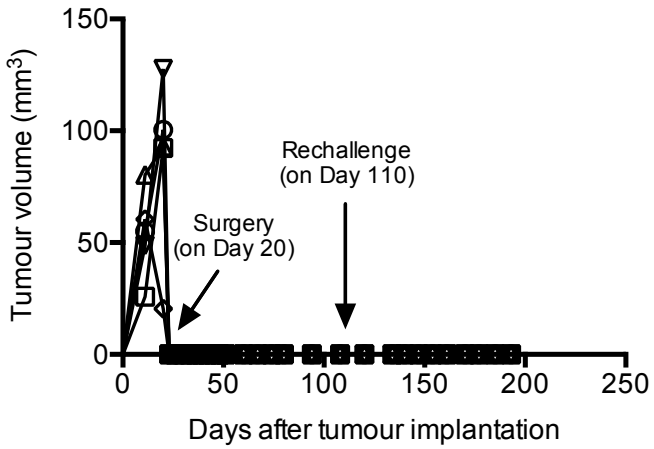
Supplementary Figure 3. Flow cytometry for $CD4^+$ T cells following neutralizing antibody treatment. **(a-c)** Representative flow cytometry dot plots of $CD3^+CD4^+$ T cells in spleen (a), TdLN (b) and EMT6 tumour (c). **(d-f)** Splenic $CD3^+CD4^+$ T cells in spleen (d), TdLN (e) and EMT6 tumour (f) 3 days after αCD4 (or isotype) antibody treatment initiation, measured by flow cytometry (single experiment; mean±SD; *t*-test).



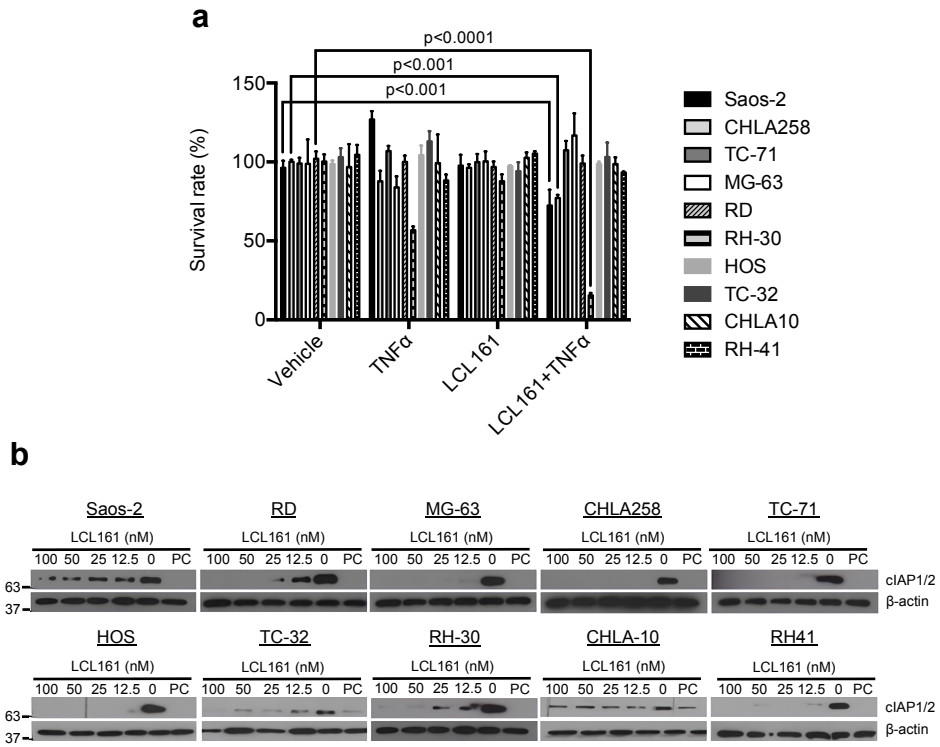
Supplementary Figure 4. LCL161 treatment leads to cIAP1/2 depletion and sensitizes EMT6 cells to TNFα-mediated cell death. **(a)** cIAP1/2 protein in EMT6 cells after treatment with LCL161 for 2 hours, detected by immunoblots (n=2 biological replicates, duplicate experiments). **(b)** EMT6 cell viability 48 hours after LCL161±recombinant TNFα (100 ng mL^{-1}) ± neutralizing antibody (TNFα or control), measured by Alamar Blue (n=3 biological replicates, duplicate experiments; mean±SD; ANOVA with Tukey's multiple comparisons test). **(c)** EMT6 cell viability 48 hours after LCL161±virus-conditioned media (VCM)±neutralizing antibody (TNFα or control), measured by Alamar Blue (n=3 biological replicates per experiment, duplicate experiments; mean±SD; ANOVA with Tukey's multiple comparisons test).



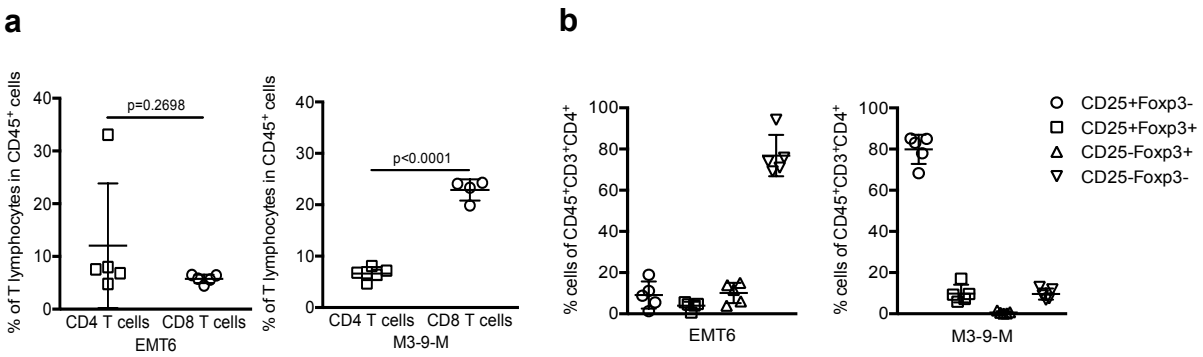
Supplementary Figure 5. Most mouse cancer cell lines are not sensitized to TNFα-mediated death by LCL161. **(a)** Cell viability 24, 48 and 72 hours after LCL161+recombinant TNFα treatment, measured by Alamar Blue (n=3 biological replicates per experiment, duplicate experiments; mean±SD; ANOVA with Tukey's multiple comparisons test). **(b)** cIAP1/2 protein in M3-9-M and 4T1 cells treated with LCL161 for 2 hours, detected by immunoblots (n=2 biological replicates per experiment, single experiment). **(c)** Tabular representation of sensitivity to LCL161+ TNFα versus immunogenicity for the mouse cancer cell lines chosen for analysis *in vivo*.



Supplementary Figure 6. EMT6 tumours are highly immunogenic. 12 day old EMT6 tumour-bearing mice underwent surgical resection and, 90 days later, rechallenged with 1e5 EMT6 cells. Tumour growth was monitored and plotted (single experiment).



Supplementary Figure 7. Most human pediatric sarcoma lines are not sensitized to TNF α -mediated death by LCL161. **(a)** Viability of human pediatric sarcoma cells 48 hours after LCL161±recombinant TNFα treatment, measured by Alamar Blue (n=3 biological replicates per experiment, duplicate experiments; mean±SD; ANOVA with Tukey's multiple comparisons test). **(b)** cIAP1/2 protein in human pediatric sarcoma cells treated with LCL161 for 2 hours, detected by immunoblots (n=2 biological replicates, single experiment).



65

66 **Supplementary Figure 8.** Divergent $CD4^+$ polarization in EMT6 vs. M3-9-M tumours. **(a)** Total
67 number of $CD8^+$ and $CD4^+$ T cells infiltrating 12 day old EMT6 or M3-9-M tumours, measured by
68 flow cytometry (duplicate experiments; mean±SD; *t*-test). **(b)** Relative number of $CD4^+$ T cells
69 expressing CD25 and/or Foxp3 polarization markers (duplicate experiment; mean±SD; ANOVA with
70 Tukey's multiple comparisons test).

71

72

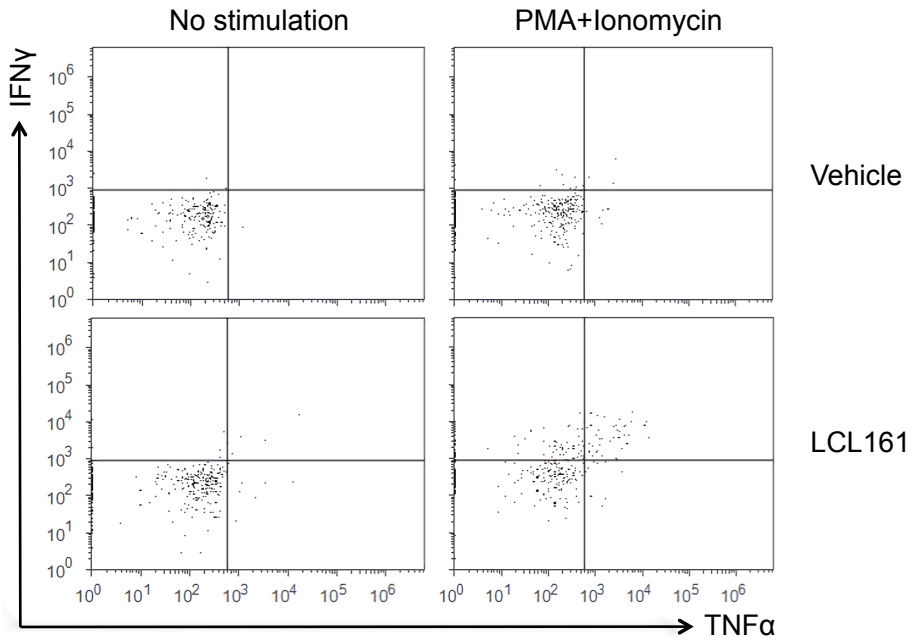
73

74

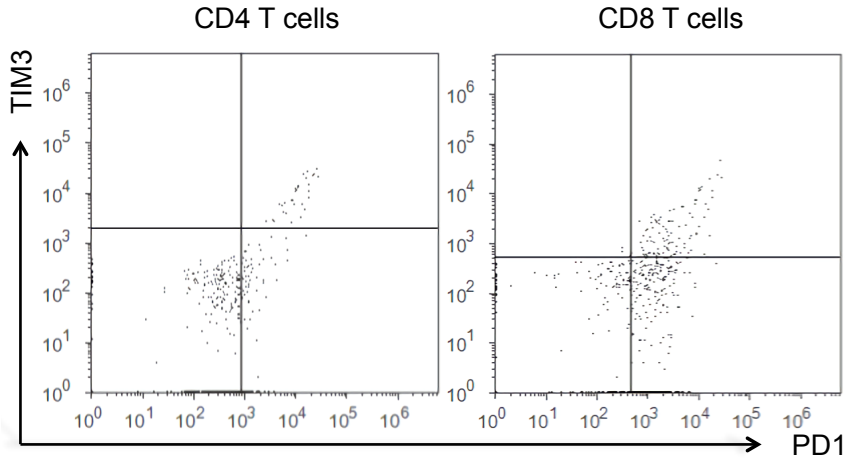
75

76

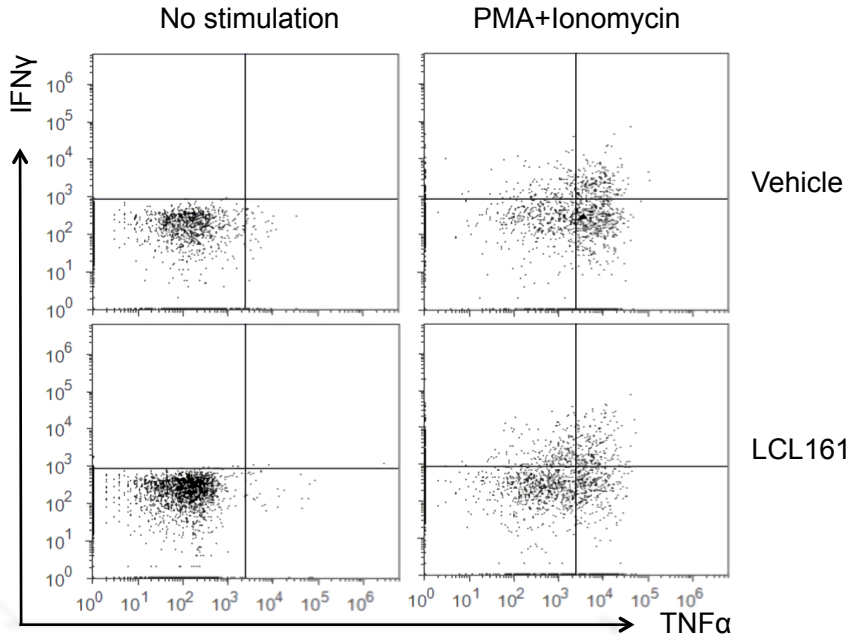
77



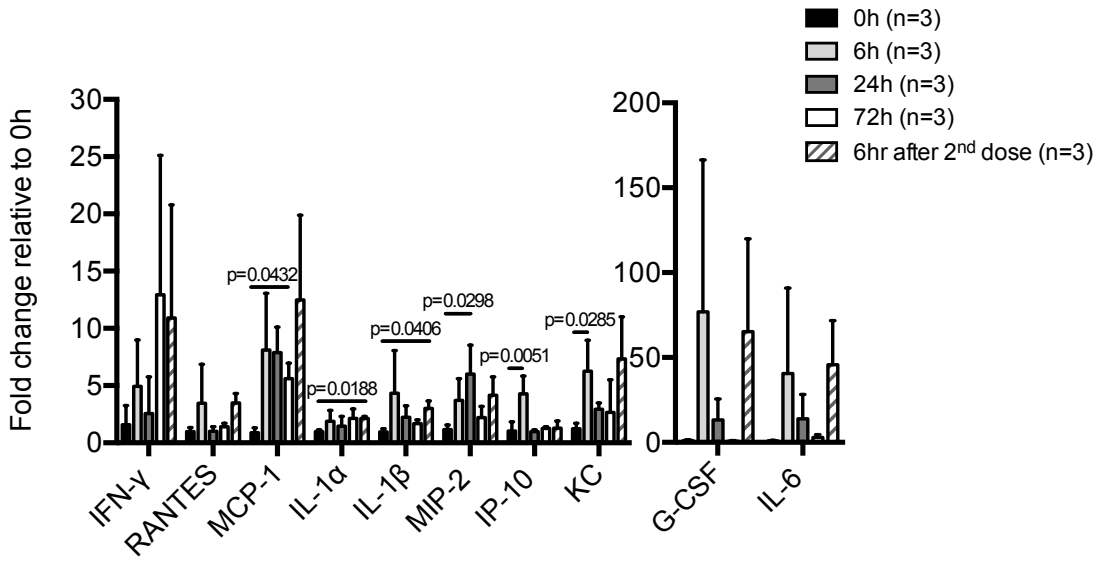
Supplementary Figure 9. Flow cytometry for intracellular cytokine staining in tumour-infiltrating $CD8^+$ T cells. Representative dot plots of IFNγ- or TNFα- or IFNγ/TNFα- positive cells within the $CD45^+CD3^+CD8^+$ leukocyte fraction isolated from EMT6 tumours.



Supplementary Figure 10. Representative dot plots of PD-1 or Tim-3 or PD-1/Tim-3- positive cells within the $CD45^+CD3^+CD8^+$ leukocyte fraction isolated from EMT6 tumours.



Supplementary Figure 11. Flow cytometry for intracellular cytokine staining in TdLN $CD8^+$ T cells. Representative dot plots of IFNγ- or TNFα- or IFNγ/TNFα- positive cells within the $CD45^+CD3^+CD8^+$ fraction isolated from EMT6 TdLN.



100

101 **Supplementary Figure 12.** LCL161 alters the cytokine milieu within EMT6$^{TNFR1-/-}$ tumours (clone 2-
102 10) toward an immunostimulatory cytokine signature. Cytokine expression within the interstitial fluid
103 of EMT6 tumours, measured by Luminex (n=3 biological replicates; single experiment; mean±SD;
104 ANOVA with Tukey's multiple comparisons test).

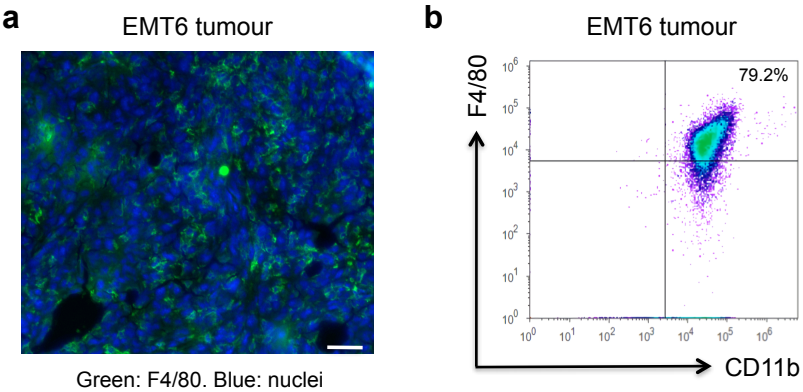
105

106

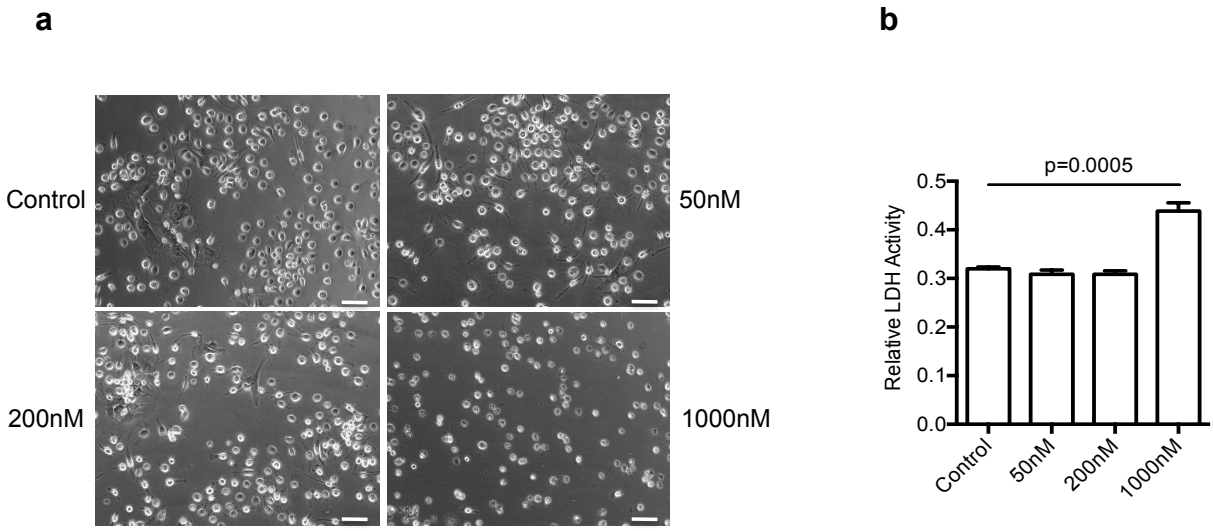
107

108

109



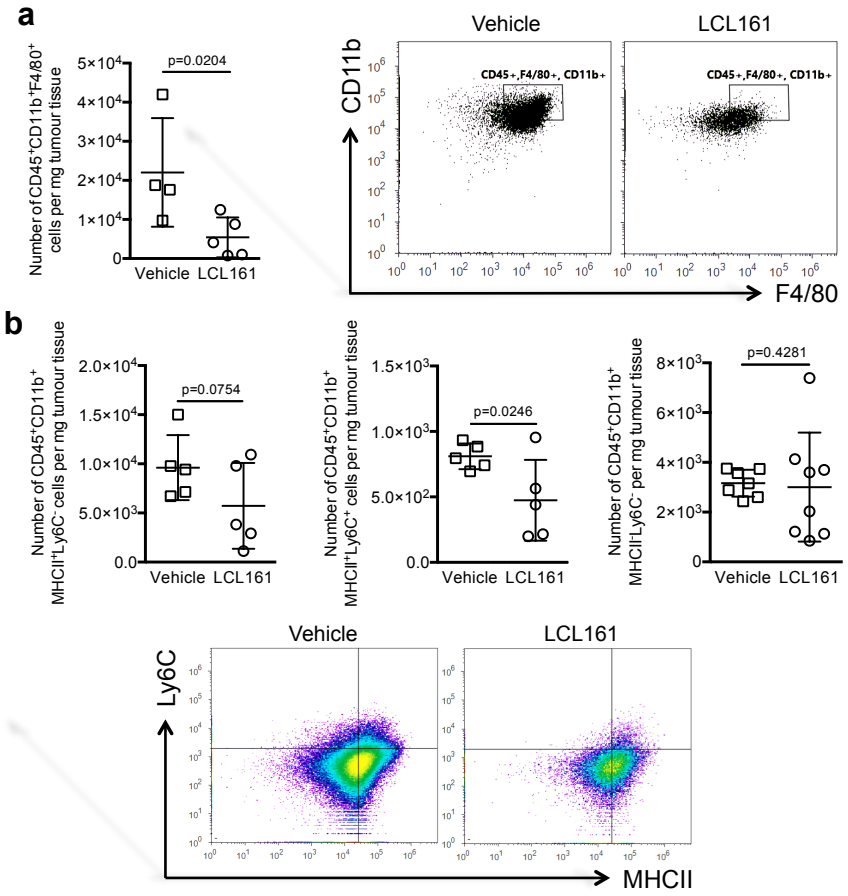
Supplementary Figure 13. EMT6 tumours are highly enriched with macrophages. **(a)** Representative immunofluorescence staining of $F4/80^{+}$ macrophages in EMT6 tumours. Green: F4/80. Blue: nuclei (DAPI). Scale bar=50μM. **(b)** Representative flow cytometry dot plot for $CD11b^{+}F4/80^{+}$ cells within the $CD45^{+}$ fraction isolated from EMT6 tumours (n=4 biological replicates per experiment, duplicate experiments).



120

121 **Supplementary Figure 14.** LCL161 treatment reduces BMDM viability. **(a)** Representative brightfield
122 images of cultured BMDM treated with LCL161 for 48 hours. Scale bar=50μM. **(b)** LDH activity in
123 BMDMs treated with LCL161 for 48 hours at the indicated concentrations (n=3 biological replicates,
124 duplicate experiments; mean±SD; ANOVA with Tukey's multiple comparisons test).

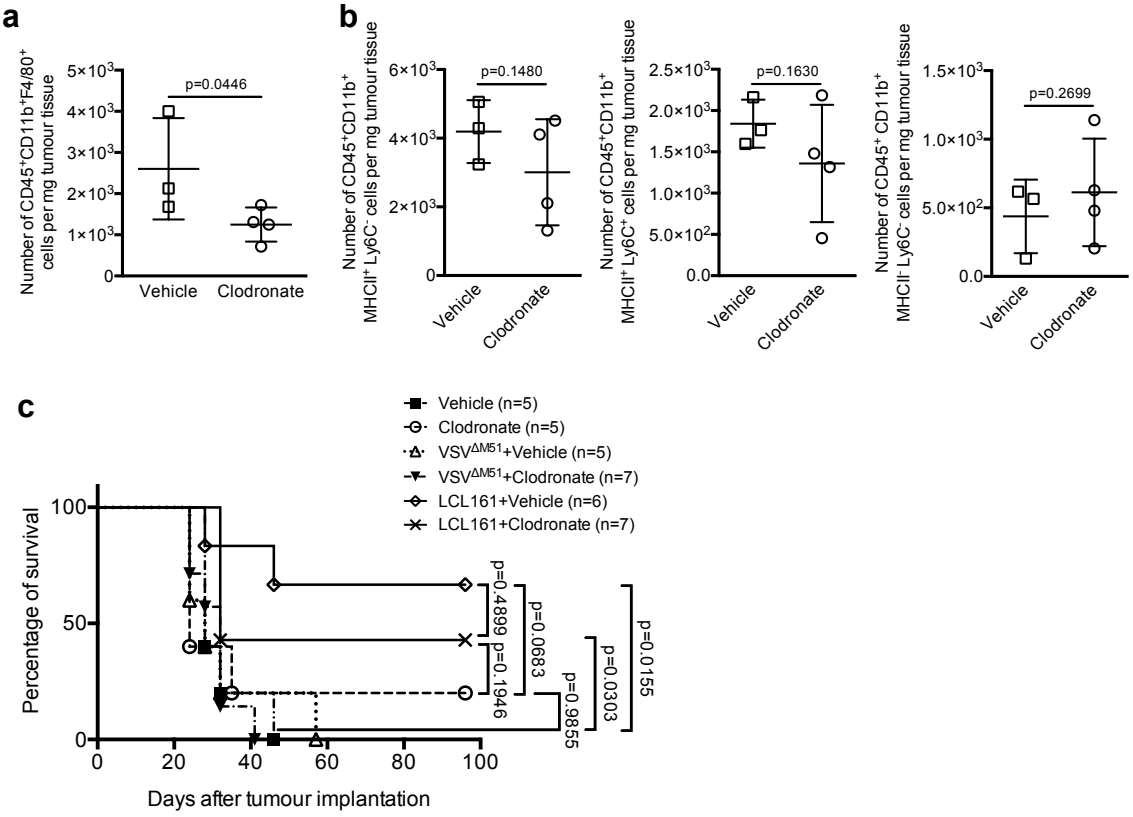
125



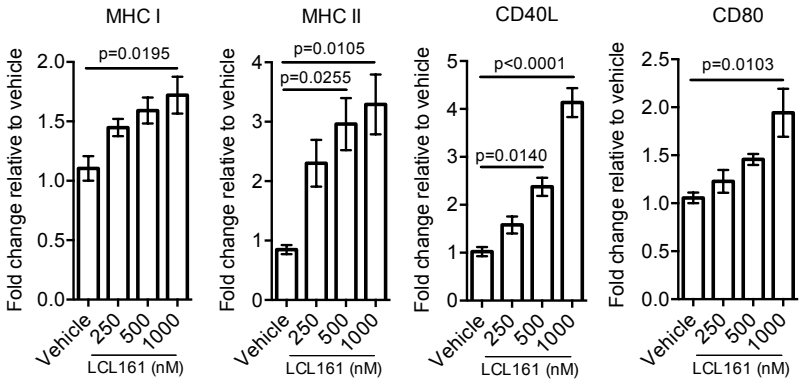
126

127 **Supplementary Figure 15.** LCL161 treatment reduces TAMs in EMT6 tumours. **(a)** ***Left panel***: Total
128 number of $CD45^{+}CD11b^{+}F4/80^{+}$ in EMT6 tumours 72 hours after LCL161 (or vehicle) treatment,
129 measured by flow cytometry (triplicate experiments; mean±SD; *t*-test). ***Right panel***: Representative dot
130 plots. **(b)** ***Top panel****:* Total number of $CD45^{+}CD11b^{+}MHC\text{-}II^{\pm}Ly6C^{\pm}$ in EMT6 tumours 72 hours after
131 LCL161 (or vehicle) treatment, measured by flow cytometry (triplicate experiments; mean±SD; *t*-test).
132 ***Bottom panel***: Representative dot plots.

133



Supplementary Figure 16. Clodronate liposome-mediated TAM depletion does not phenocopy LCL161 therapy. **(a)** Total number of $CD45^+CD11b^+F4/80^+$ in EMT6 tumours 7 days after CL (or vehicle) treatment, measured by flow cytometry (single experiment; mean±SD; *t*-test). **(b)** Total number of $CD45^+CD11b^+MHC\text{-}II^{\pm}Ly6C^{\pm}$ in EMT6 tumours 7 days after CL (or vehicle) treatment, measured by flow cytometry (single experiment; mean±SD; *t*-test). **(c)** Overall survival of EMT6 tumour bearing mice treated with the indicated therapies beginning on day 12. (single experiment; log-rank test)



143

144 **Supplementary Figure 17.** LCL161 causes upregulation of MHC and co-stimulatory proteins on
145 macrophages. BMDMs were treated with the indicated concentrations of LCL161 (or vehicle) for 20
146 hours prior to flow cytometry for MHC-I, MHC-II, CD40L and CD80 (n=3 biological replicates,
147 duplicate experiments; mean±SD; ANOVA with Tukey's multiple comparisons test).

148

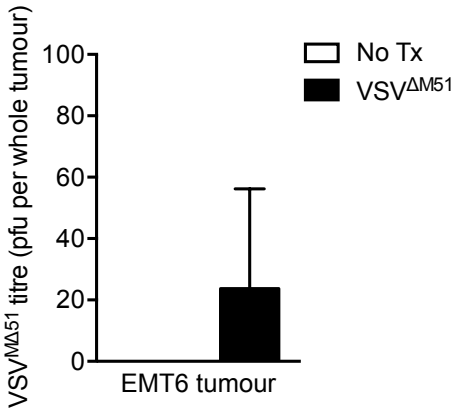
149

150

151

152

153



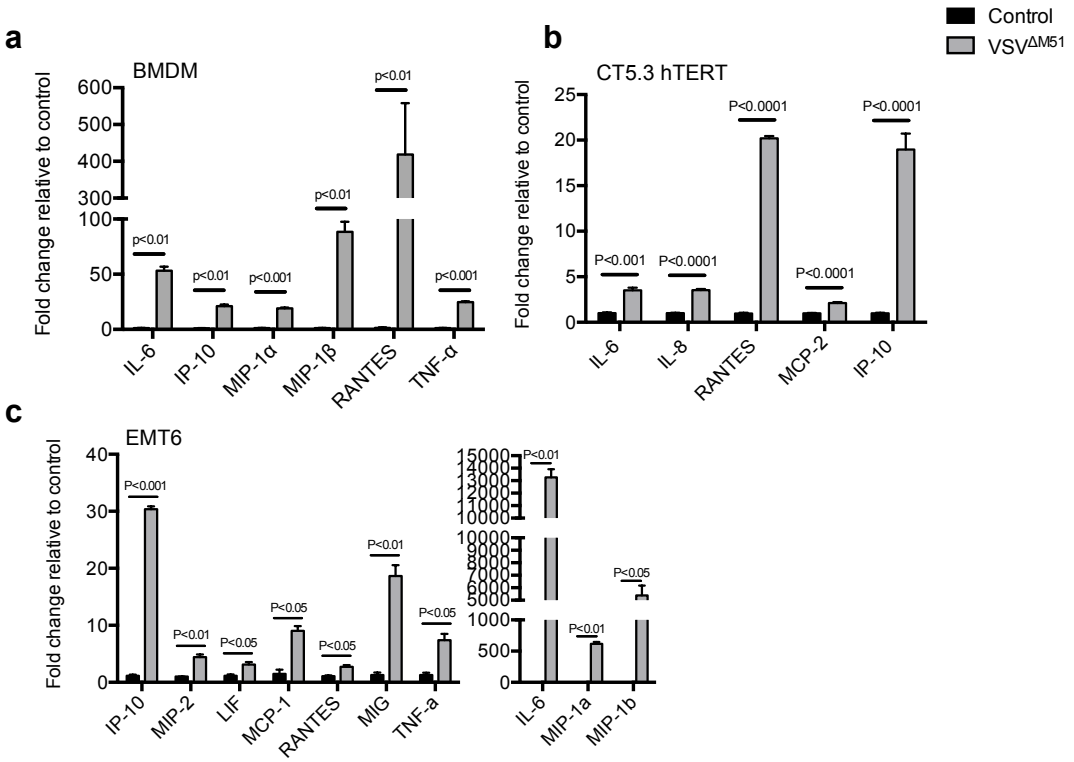
154

155 **Supplementary Figure 18.** Systemic delivery of $VSV^{\Delta M51}$ leads to a small infection of EMT6
156 tumours. 12 day old EMT6 tumour-bearing mice were treated with $VSV^{\Delta M51}$($1x10^8$ PFU) i.v. and 12
157 hours later infectious particles measured in tumours by plaque assay (n=3 biological replicates; single
158 experiment; mean±SD).

159

160

161



162

163 **Supplementary Figure 19.** VSV$^{\Delta M51}$ elicits cytokine secretion from cancer and normal cells *in vitro*.

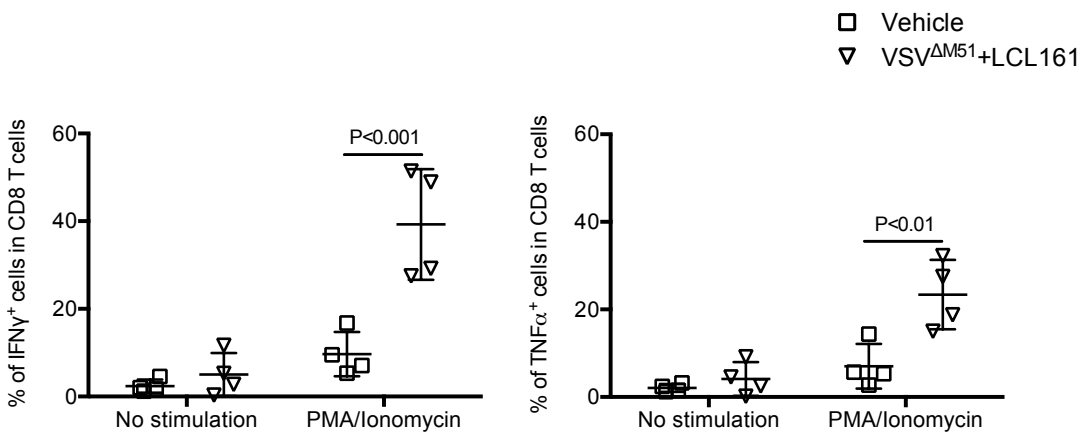
164 **(a-c)** Immunostimulatory cytokines measured from EMT6 (**a**, MOI=0.1, 48 hpi), BMDM (**b**, MOI=10,

165 24 hpi) and cancer-associated fibroblasts (CT5.3 hTERT, **c**, MOI=0.1, 48 hpi) culture after infection

166 with VSV$^{\Delta M51}$ (n=3 biological replicates, single experiment; mean±SD; *t*-test).

167

168



169

170 **Supplementary Figure 20.** LCL161 rejuvenates tumour-infiltrating T cells in mice bearing

171 EMT6$^{TNFR1-/-}$ tumours. Intracellular staining for IFNγ or TNFα within CD8^{+} T cells isolated from

172 EMT6$^{TNFR1-/-}$ tumours (clone 2-10) and stimulated with PMA and ionomycin *ex vivo*, measured by flow

173 cytometry (single experiment; mean±SD; ANOVA with Bonferroni's multiple comparisons test).

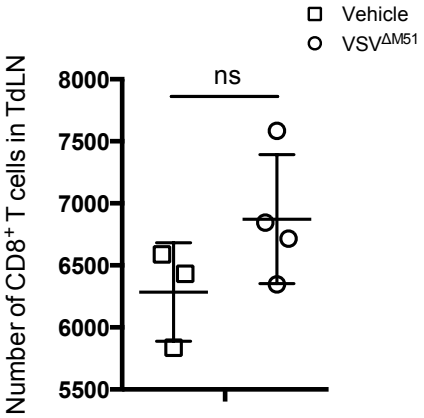
174

175

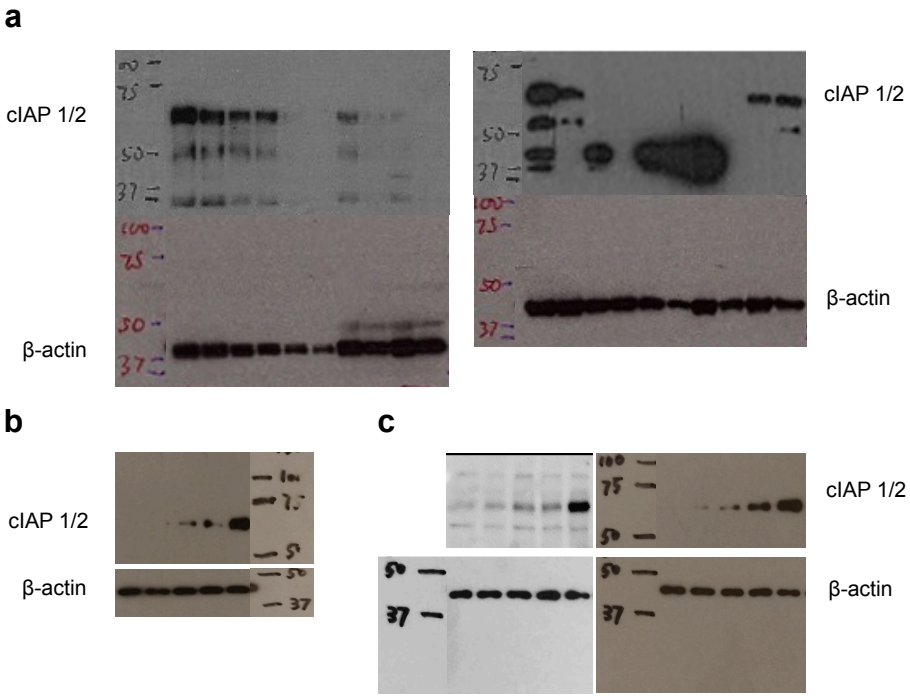
176

177

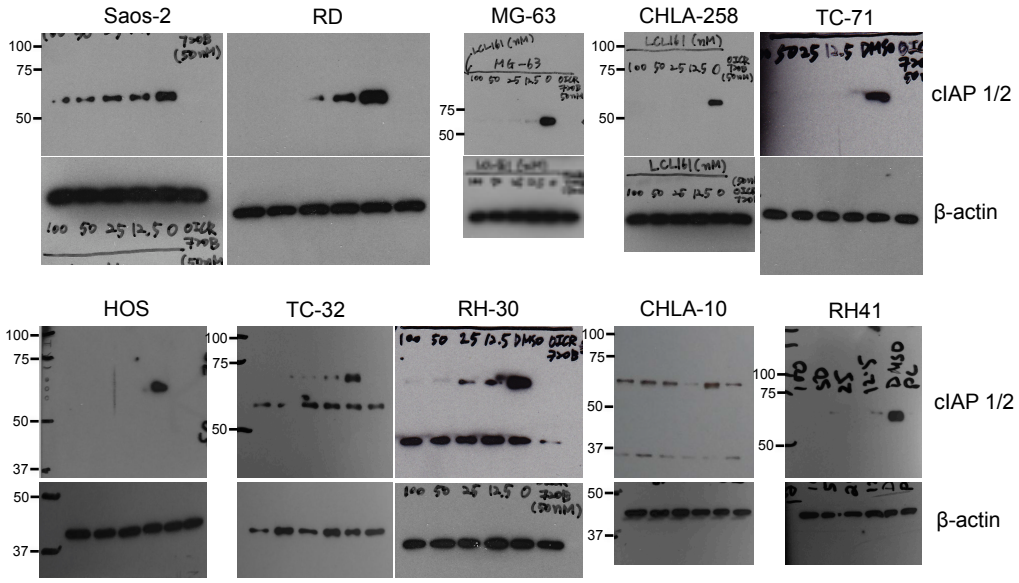
178



Supplementary Figure 21. VSV$^{\Delta M51}$ has no effect on the number of CD8^{+} T cells within the TdLN. CD8^{+} T cells within EMT6 TdLN 7 days after LCL161 (or vehicle) treatment initiation, measured by flow cytometry (duplicate experiments; mean±SD; *t*-test).



Supplementary Figure 22. Whole blots shown in (a) Supplementary Fig. 1c, (b) Supplementary Fig. 4a, (c) and Supplementary Fig. 5b



Supplementary Figure 23. Whole blots shown in Supplementary Fig. 7

Gene of Interest	Annealing Temp	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	55.7	CACGGCAAATTCAACGGCACAGT	TGGGGGCATCGGCAGAAGG
IFN α	57	CTTCCACAGGATACTGTGTACCT	TTCTGCTCTGACCACCCTCCC
IFN β	57	ATGAGTGGTGGTTGCAGGC	TGACCTTTCAAATGCAGTAGATTCA
IRF7	59	AGCAAGACCGTGTTTACGAC	AGTGCTGAAGTCGAAGATGG
OAS	63.3	GCCATTGCACGCTCGCCTACTAC	CTCCTGCCATCCGGGTTTTTCA
STAT1	55	CTGAATATTTCCCTCCTGGG	TCCCGTACAGATGTCCATGAT
Mx2	57	CCTGCCTGCCATCGCTGTC	GCCTCTCCACTCCTCTCCCTCATT
iNOS	55	TTTGCTTCCATGCTAATGCGAAAG	GCTCTGTTGAGGTCTAAAGGCTCCG
Arg1	60.9	AGGGTTACGGCCGGTGGAGAG	CCCCTCCTCGAGGCTGTCCTTTT

Supplementary Table 1. Annealing temperatures and primer sequences used each gene specific semi-quantitative RT-PCR amplification