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Supplementary information

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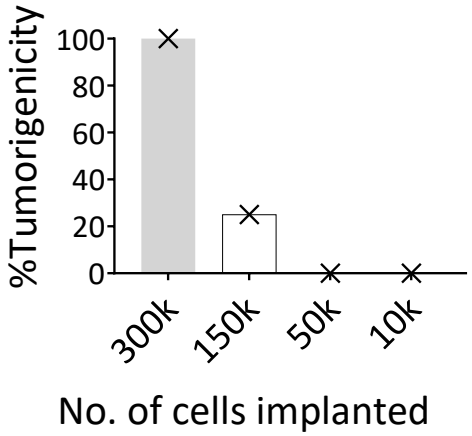
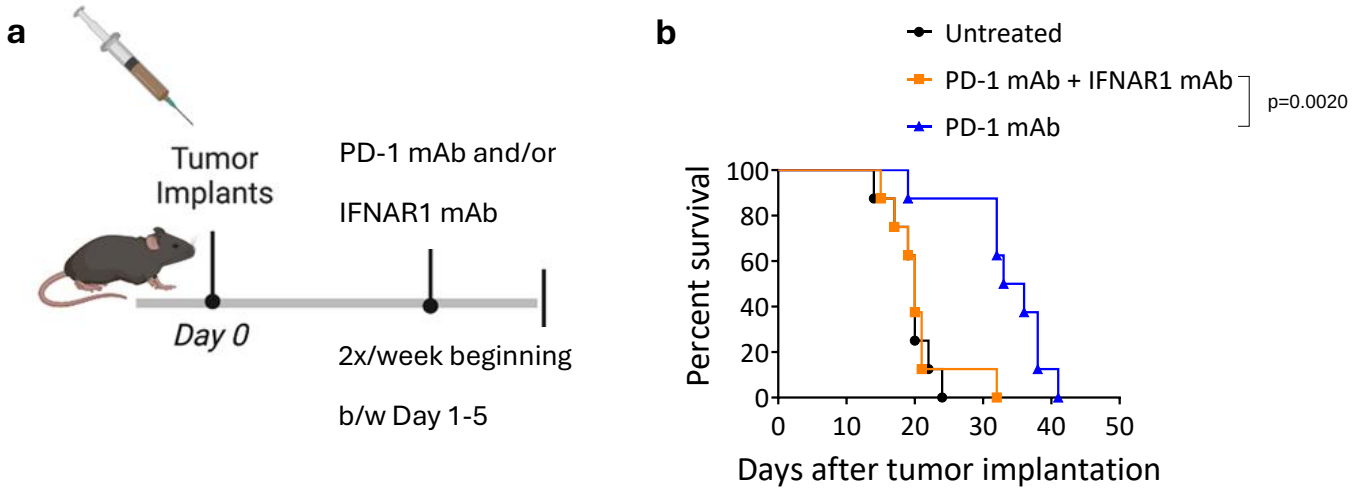
Sensitization of tumours to immunotherapy by boosting early type-I interferon responses enables epitope spreading

In the format provided by the authors and unedited

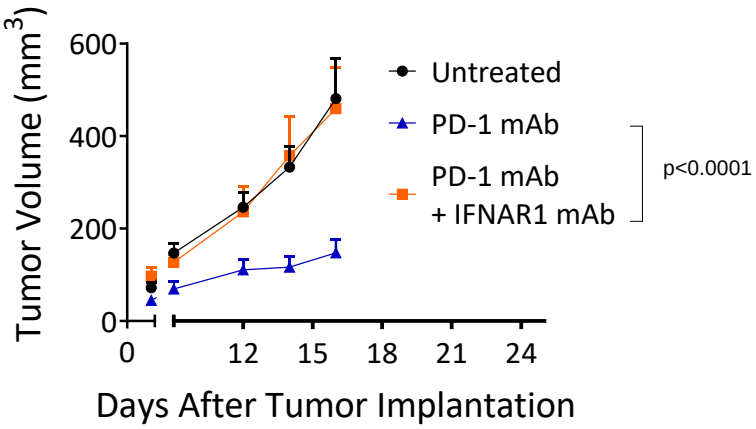
Supplementary Fig. 1, Tumourigenicity of intravenously implanted melanomas. Various cell doses (300k, 150k, 50k, and 10k cells) of B16F10-OVA melanoma were administered intravenously (i.v.) into naïve C57Bl/6 mice (n=5/group). Lungs were harvested on day 14 to determine the percentage of animals with lung nodules.



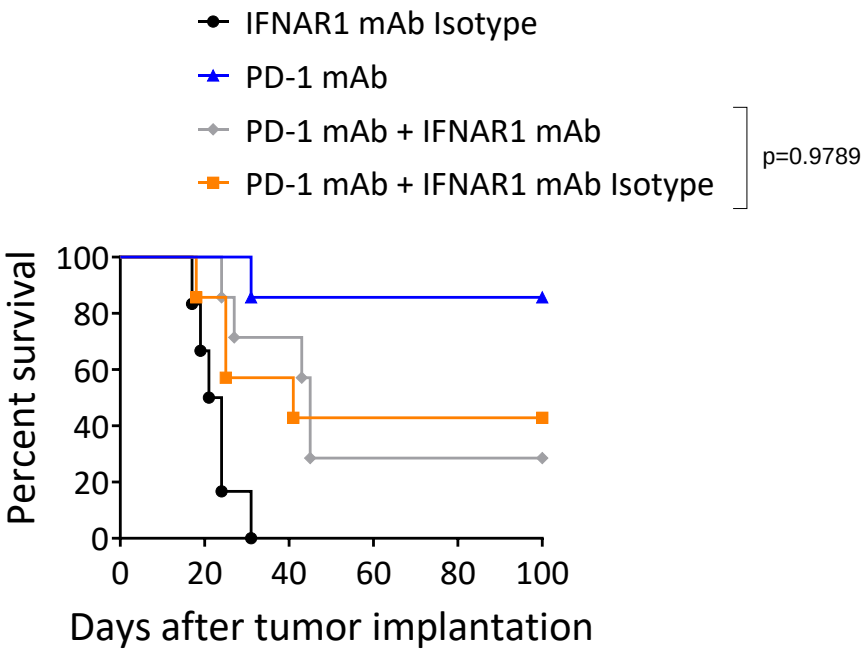
Supplementary Fig. 2: Survival outcomes of GL261 bearing animals treated with ICIs and IFNAR1 blocking antibodies: a, Treatment schema of Supplementary Fig 2-5. **b,** GL261 was implanted intracranially into C57Bl/6 mice (n=8/group) who were treated intraperitoneal (i.p.) with PD-1 mAb (beginning Day 5) administered with or without IFNAR1 mAb (beginning Day 1). Statistical significance determined by log-rank test.



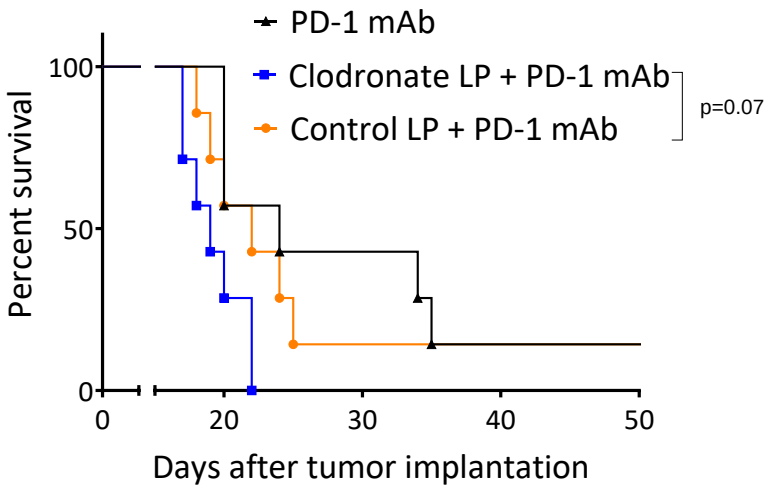
Supplementary Fig. 3: Subcutaneous tumour volumes of mice bearing B16F10-OVA treated with ICIs and IFNAR1 blocking antibodies: B16F10-OVA tumours were implanted subcutaneously in C57Bl/6 mice (n=8/group) and experimental animals were subjected to biweekly i.p. systemic PD-1 mAb (beginning day 5) administered with or without IFNAR1 mAb (beginning Day 1). Statistical significance determined by mixed effect model/two-way ANOVA.



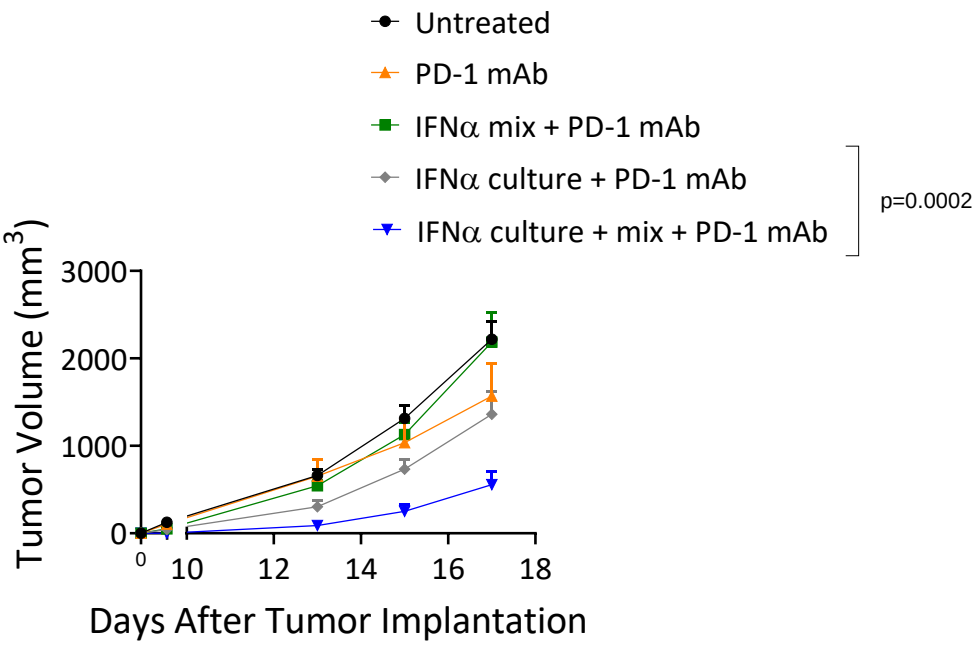
Supplementary Fig. 4: Survival outcomes of GL261 bearing animals treated with ICIs, isotypes and IFNAR1 blocking antibodies: Kaplan-Meier survival curves of C57Bl/6 mice (n=6-7) intracranially implanted with GL261 glioma cells, treated with i.p. PD-1 mAb alone with or without IFNAR1 mAbs or isotype control, with day 1 as a starting treatment date followed by biweekly injection from day 2 to day 21.



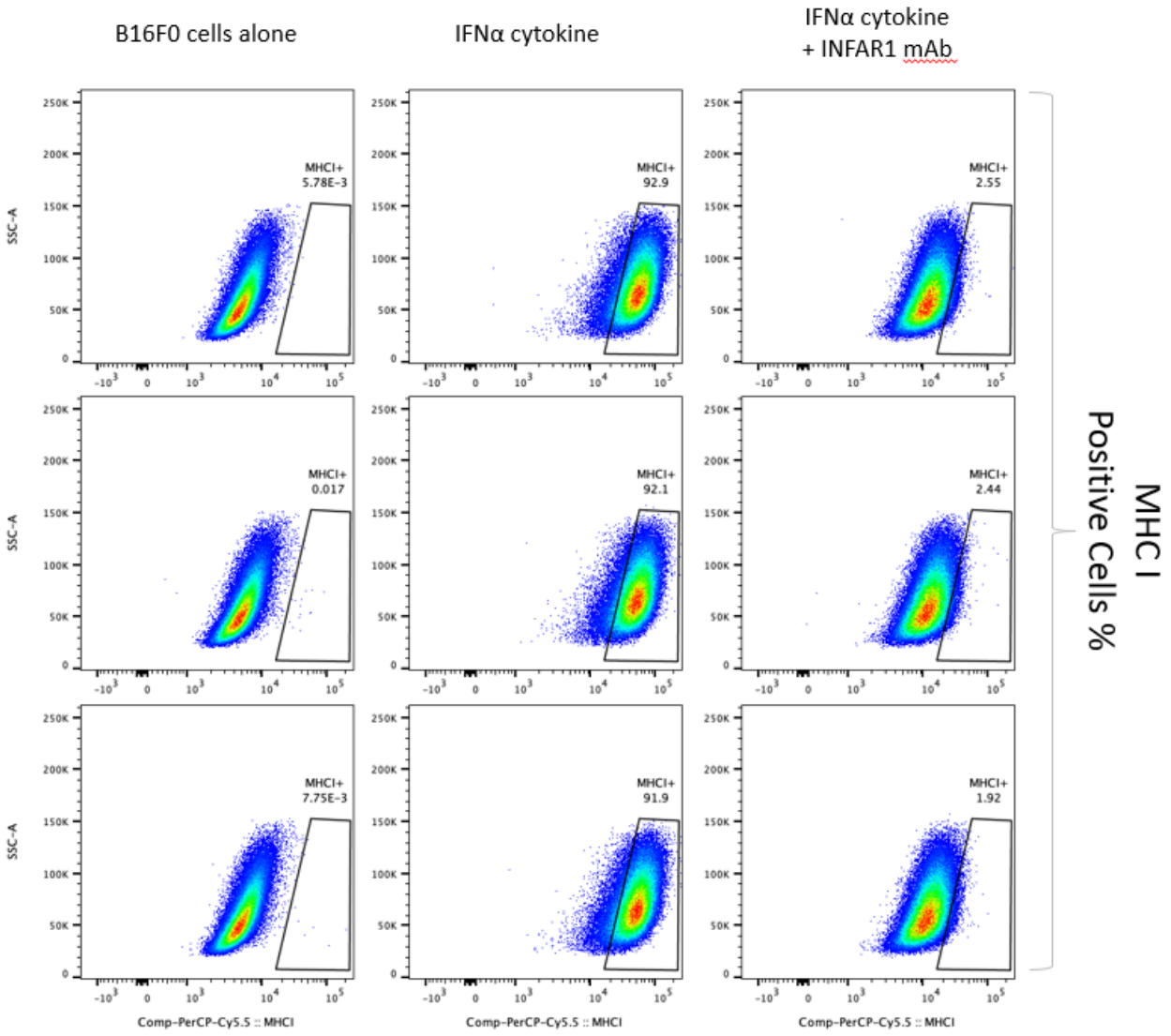
Supplementary Fig. 5: Survival outcomes of GL261 bearing animals treated with ICIs and clodronate liposomes: Kaplan-Meier survival curve for C57Bl/6 mice (n=7) implanted intracranially with GL261 glioma cells, treated with clodronate or control liposomes (LP, 200µL), administered intravenously 24 hours prior to tumour implantation. PD-1 mAb was subsequently administered twice weekly (beginning on day 5). Statistical significance determined by log-rank test.



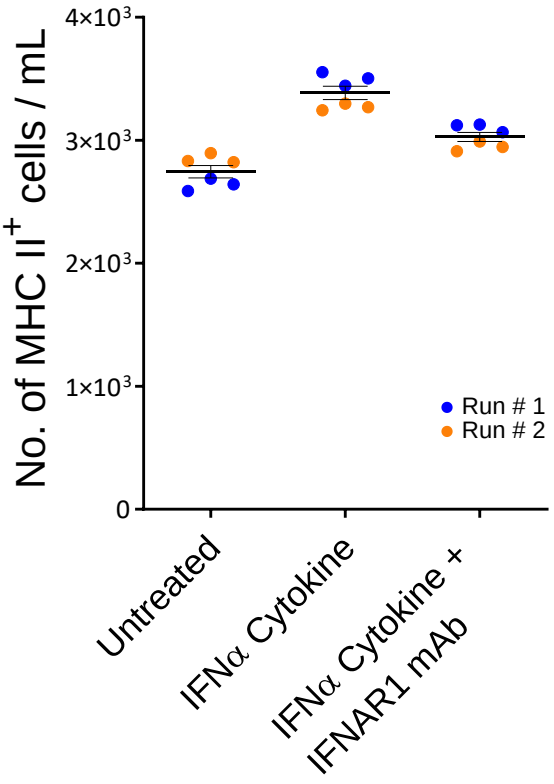
Supplementary Fig. 6: Early IFN treatment sensitizes ICI response. Tumour growth curve for C57Bl/6 mice (n=7/group) implanted with B16F0 melanomas. IFNα was either directly added in co-culture with B16F0 cells (IFNα culture) or mixed with B16F0 cells (IFNα mix) prior to tumour implantation. All groups received subcutaneous implantation of cells suspended in a methyl-cellulose solution. The mice received an initial dose of PD-1 mAb on day 1 followed by half the dosage twice weekly for three weeks. IFNα mix corresponds to mixture of IFNα and B16F0 tumour cells prior to implantation; IFNα culture corresponds to IFNα co-cultured with B16F0 tumour cells prior to implantation; and IFNα culture + mix corresponds to co-culture of IFNα with B16F0 tumour cells followed by mixture of B16F0 tumour cells with IFNα prior to animal inoculation. Statistical significance determined by mixed effect model/two-way ANOVA.



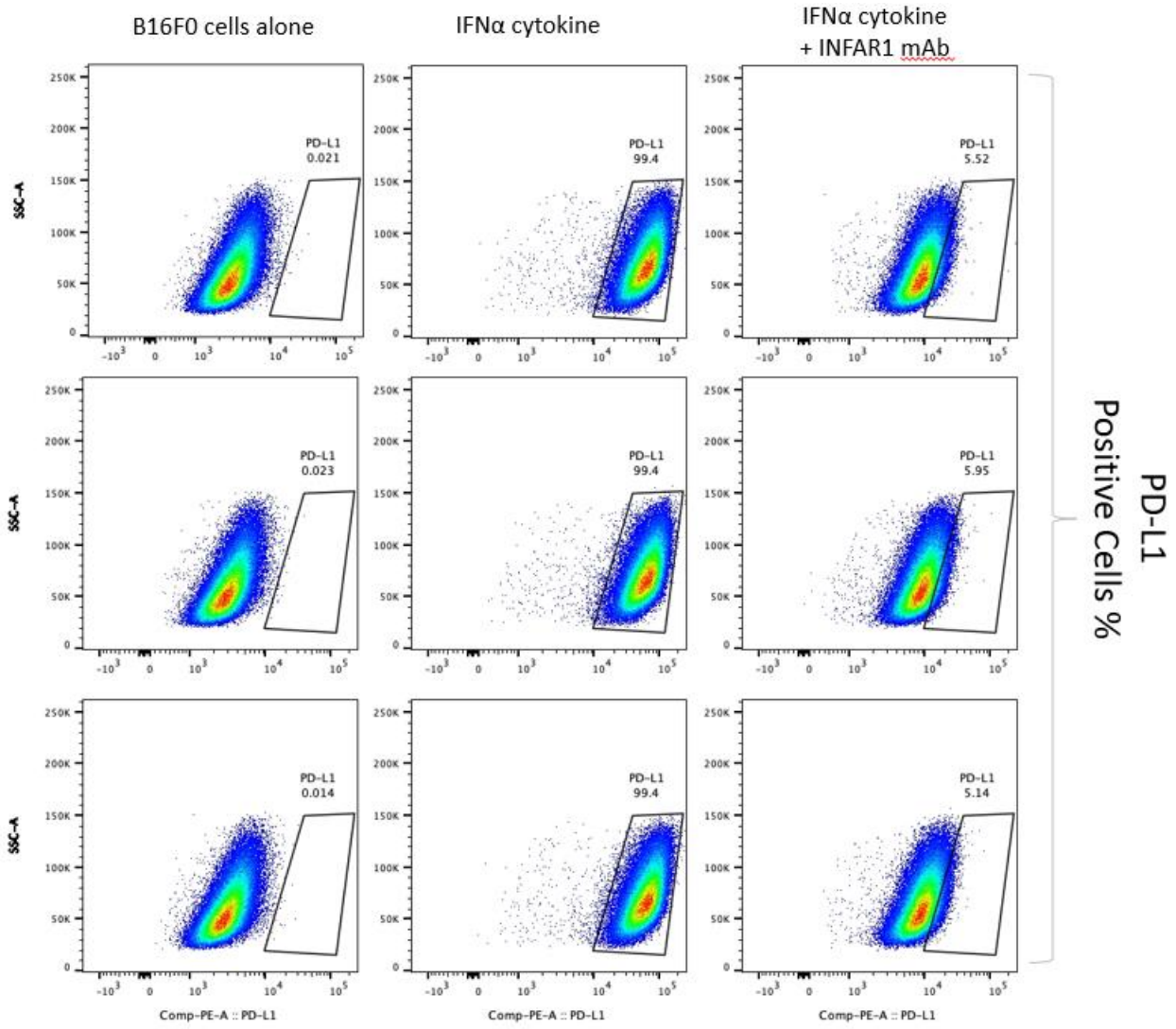
Supplementary Fig. 7: MHC-I expression on B16F0: Flow cytometric analysis of MHC-I expression from B16F0 tumour cells following 24h co-culture with IFNα cytokine or IFNα cytokine + IFNAR1 mAb.



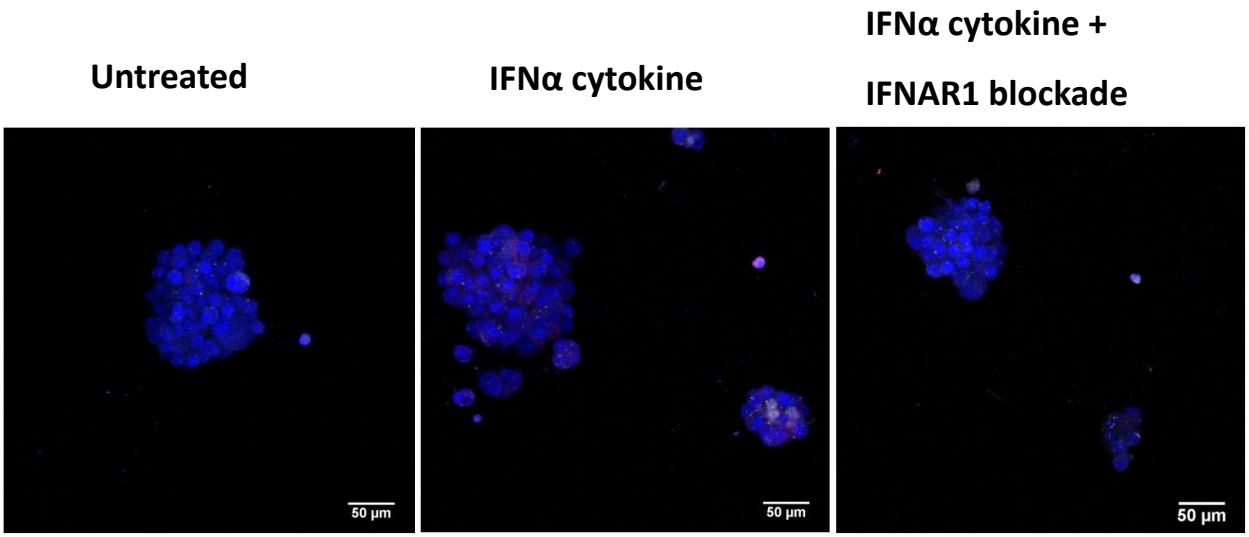
Supplementary Fig. 8: MHC-II expression on B16F0 tumours: B16F0 melanomas were co-cultured for 24h with IFNα cytokine or IFNα cytokine + IFNAR1 mAb, and the number of MHC-II positive cells were plotted using flow cytometry (Biological replicates are shown in different colors, n=2; technical replicates, n=3).

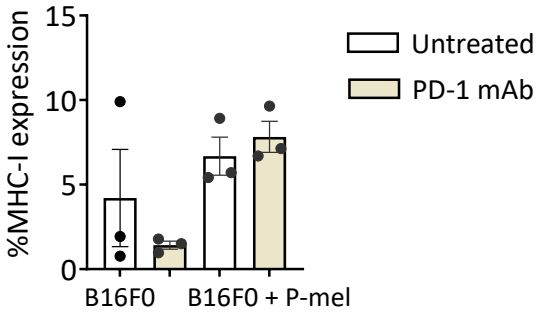


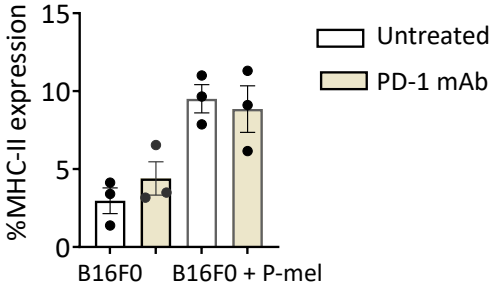
Supplementary Fig. 9: PD-L1 expression on B16F0 tumours: Flow cytometric analysis for PD-L1 expression from B16F0 tumours following 24h co-cultured with IFNα cytokine or IFNα cytokine + IFNAR1 mAb.



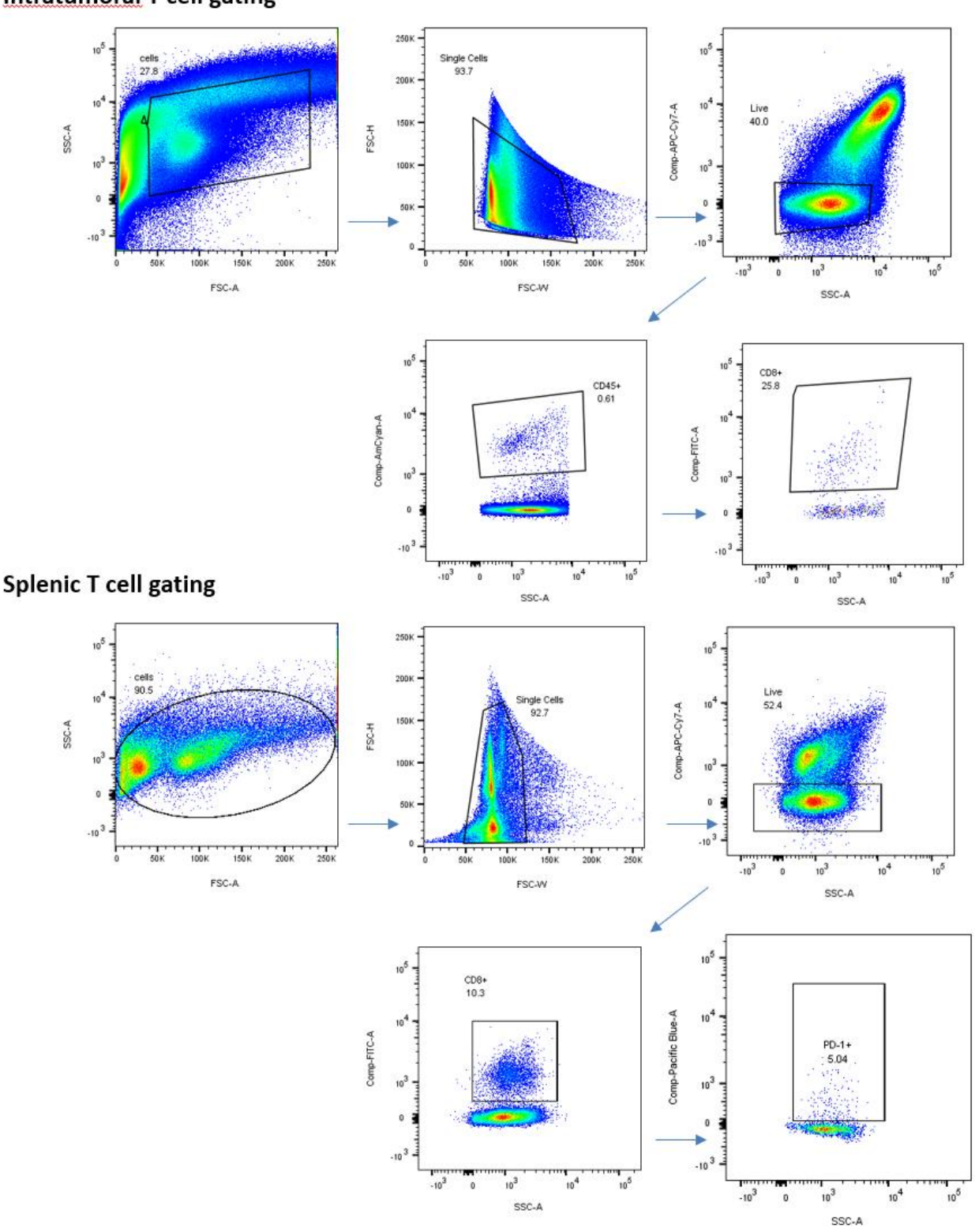
Supplementary Fig. 10: MHC expression on K7M2 tumouroids and B16F0 cells: *Top*, Merged (pink) Immunofluorescent images depicting MHC-I (Gold), MHC-II (FITC), and PD-L1 (TRITC) expression in 3D spheroids of K7M2 pulmonary osteosarcomas, *treated* with IFNα cytokine alone or a combination of IFNα cytokine and IFNAR1 mAb. *Bottom,* MHC-I/II expression by flow cytometry from B16F0 cells (3 technical replicates) grown in vitro and left untreated or treated with PD-1 mAb, both with or without the addition of P-mel T cells. Error bars are reported as the standard error of the mean.



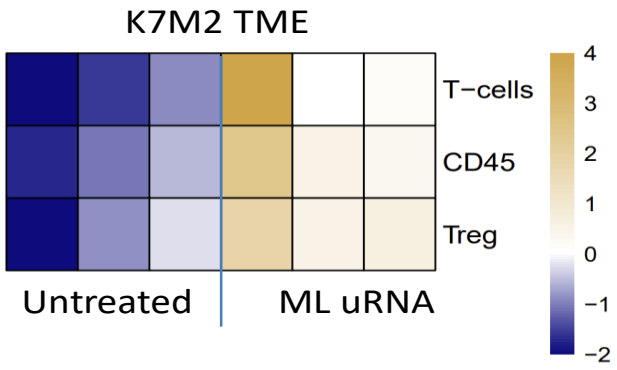




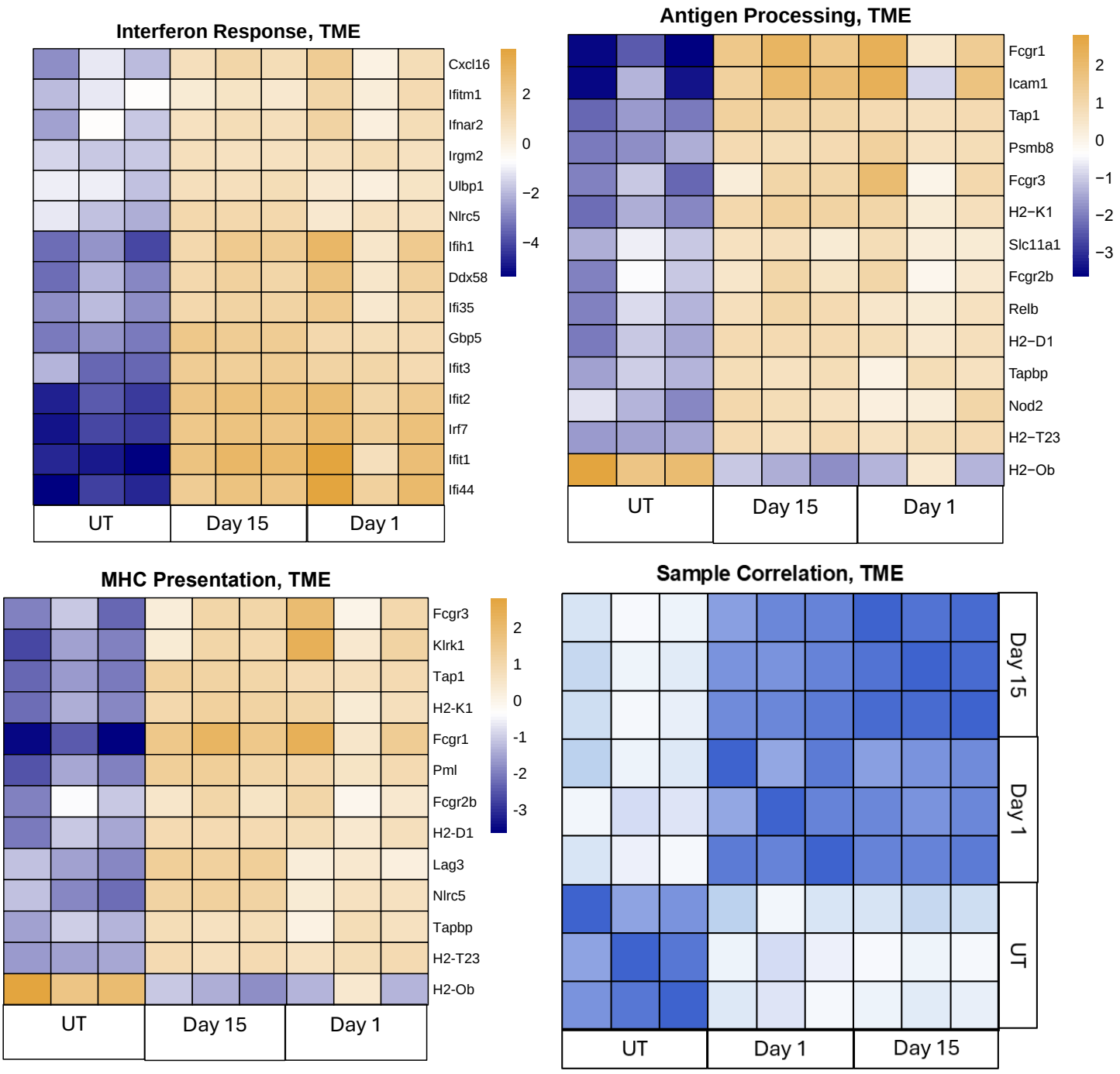
Supplementary Figure 11: Example of gating strategy for intratumoural (top) and splenic T cells (bottom)



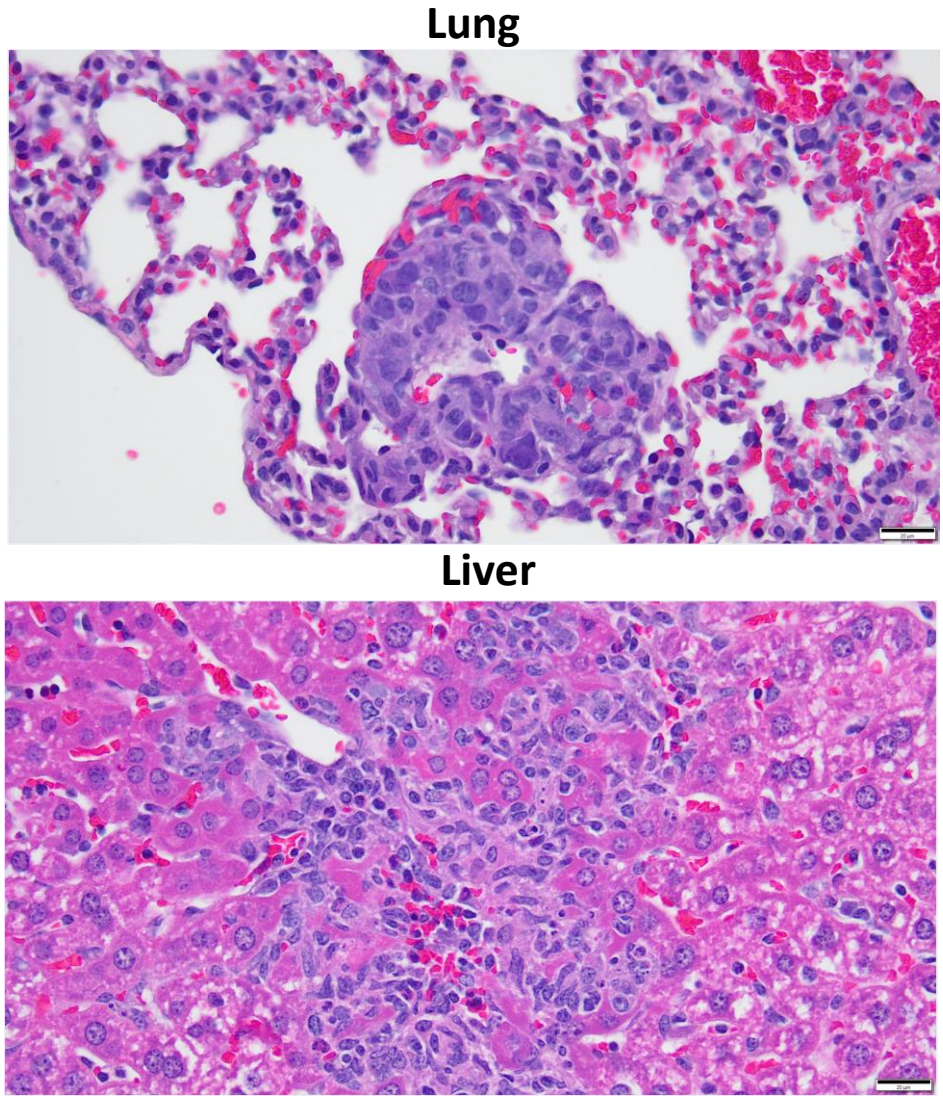
Supplemental Figure 12: ML uRNA elicits adaptive response in TME with increased Tregs. Balb/c mice (n=3/group) bearing pulmonary K7M2 tumours were left untreated or vaccinated i.v. with serial ML uRNA encoding for pp65 on days 5, 7, 9, 16, 23, 30 before lungs were harvested for evaluation of gene signatures for T cells, CD45+ cells and regulatory T cells.



Supplementary Figure 13: Early versus Late treatment with ML uRNA on intratumoural changes in the microenvironment. C57Bl/6 mice (n=3/group) bearing pulmonary B16F0 tumours were left untreated or treated with early (starting Day 1) or late (starting Day 15) administration of ML uRNA before established tumours were harvested for analysis of the tumour microenvironment (TME) by Nanostring. Sample correlation heatmap in *Bottom-right* demonstrates strong concordance between intratumoural effects of early and late treatment with ML uRNA.



Supplementary Fig. 14: H&E of liver/lung tumours from K7M2 i.v. inoculated mice.



Supplementary Figure 15: Example of gating strategy for CD4 and CD8 T cells stained for AIM activation markers in shown in Fig. 8b

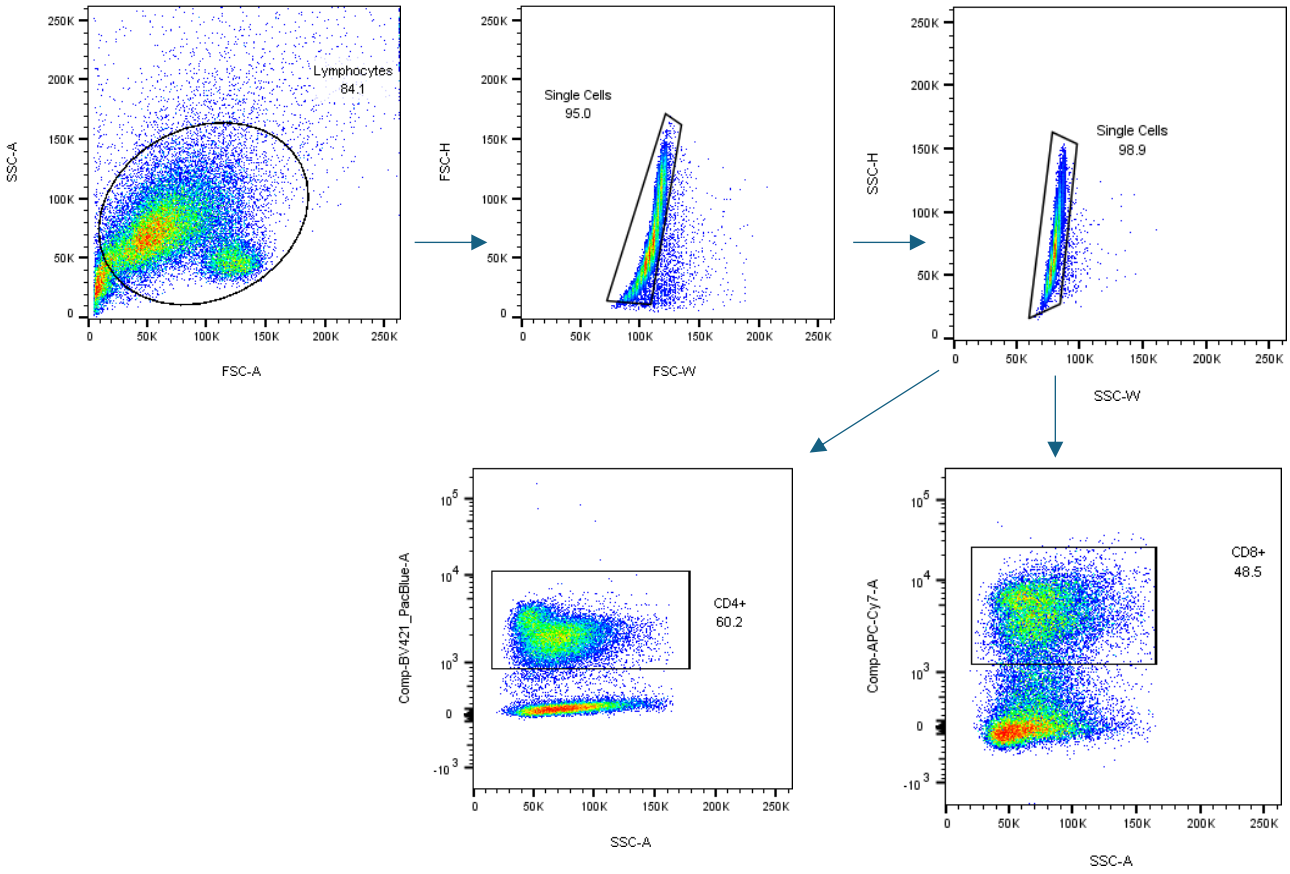


Table 1: Blinded scoring of organs from K7M2 i.v. inoculated untreated (UT) versus ML uRNA treated Balb/c mice. Organs harvested within 24h of i.v. ML uRNA infusion for acute toxicity analysis.

Case ID	Organ	Treatment	Edema (0=< 5%, 1=6-25%, 2=26-50%, 3>50%)	Interstitial Infiltrate (mild, moderate, severe)	Neutrophils/5 hpf ((x40X),0=<0-9, 1=10-19 hpf, 2=20-39, 3=>40)	Apoptotic cells/5 hpf ((x40x); 0=<5, 1=6-10 hpf, 2=11-20, 3=>20)	Additional Comments
1	Brain	UT	0	0	0	0	
2			0	0	0	0	
3			0	0	0	0	
4			0	0	0	0	
5		ML uRNA	0	0	0	0	
6			0	0	0	0	
7			0	0	0	0	
8			0	0	0	0	
9	Spleen	UT	0	0	0	0	
10			0	0	0	0	
11			0	0	0	0	
12			0	0	0	0	
13		ML uRNA	0	0	0	0	
14			0	0	0	0	
15			0	0	0	0	
16			0	0	0	0	
17	Lungs and Liver	UT	0	mild	1	1	in both the liver and lung there is metastatic disease. Inflammation and apotosis is associated with tumor.
18			0	mild	0	1	in both the liver and lung there is metastatic disease. Inflammation and apotosis is associated with tumor.
19			0	mild	1	0	in both the liver and lung there is metastatic disease. Inflammation and apotosis is associated with tumor.
20			0	mild	1	0	in both the liver and lung there is metastatic disease. Inflammation and apotosis is associated with tumor.
21		ML uRNA	0	0	0	0	no disease in lung or liver
22			0	0	0	0	no disease in lung or liver
23			0	0	0	0	no disease in lung or liver
24			0	0	0	0	lung has one focus of tumor/no disease in liver
25	Heart and Kidneys	UT	0	0	0	0	
26			0	0	0	0	
27			0	0	0	0	
28			0	0	0	0	
29		ML uRNA	0	0	0	0	
30			0	0	0	0	
31			0	0	0	0	

Spleen- With no significant histopathologic alteration. No architectural or cytologic differences

Brain- Cytoarchitecture is preserved. Lamination is intact, No reactive gloss, inflammation including microglia.

Heart- Shows no evidence of acute or chronic inflammation, reactive cellular hypertrophy, or fibrosis.

Lung- No bronchial fibrosis or acute/chronic inflammation. No vasculitis. No reactive type II hyperplasia, acute or organizing pneumonia-like change, or hyaline membrane formation.

Liver- Chronic inflammation is mainly interstitial. No evidence of zonal necrosis, fibrosis, periductal chronic inflammation, interstitial or bridging fibrosis seen in any of the livers.

Kidney- Shows no evidence of glomerulosclerosis, arteriopathy, interstitial fibrosis or significant interstitial acute or chronic inflammation.