

Supplementary Information

Panpan Dai^{1#}, Yishuang Sun^{1,2#}, Zhengrong Huang^{3#}, Yu-Tong Liu^{4#}, Minling Gao^{1,2}, Hai-Ming Liu⁴, Jie Shi^{1,2}, Chuan He^{1,2}, Bolin Xiang^{1,2}, Yingmeng Yao^{1,2}, Haisheng Yu^{1,2}, Gaoshan Xu^{1,2}, Lijun Kong^{1,2}, Xiangling Xiao^{1,2}, Xiyong Wang^{1,2}, Xue Zhang^{1,2}, Wenjun Xiong^{1,2}, Jing Hu¹, Dandan Lin⁵, Bo Zhong^{2,6}, Gang Chen^{4,7,8*}, Yan Gong^{3*}, Conghua Xie^{1,9*}, Jinfang Zhang^{1,2*}

Affiliation

¹Department of Radiation and Medical Oncology, Medical Research Institute, Frontier Science Center of Immunology and Metabolism, Hubei Key Laboratory of Tumor Biological Behavior, Hubei Provincial Clinical Research Center for Cancer, Zhongnan Hospital of Wuhan University, Wuhan University, Wuhan 430071, China

²State Key Laboratory of Metabolism and Regulation in Complex Organisms, College of Life Sciences, Taikang Center for Life and Medical Sciences, Wuhan University, Wuhan, China

³Tumor Precision Diagnosis and Treatment Technology and Translational Medicine, Hubei Engineering Research Center, Zhongnan Hospital of Wuhan University, Wuhan 430071, China

⁴State Key Laboratory of Oral & Maxillofacial Reconstruction and Regeneration, Key Laboratory of Oral Biomedicine Ministry of Education, Hubei Key Laboratory of Stomatology, School & Hospital of Stomatology, Wuhan University, Wuhan 430079, China

⁵Cancer Center, Renmin Hospital of Wuhan University, Wuhan 430061, China

⁶Medical Research Institute, Frontier Science Center for Immunology and Metabolism, Wuhan University, Wuhan 430071, China

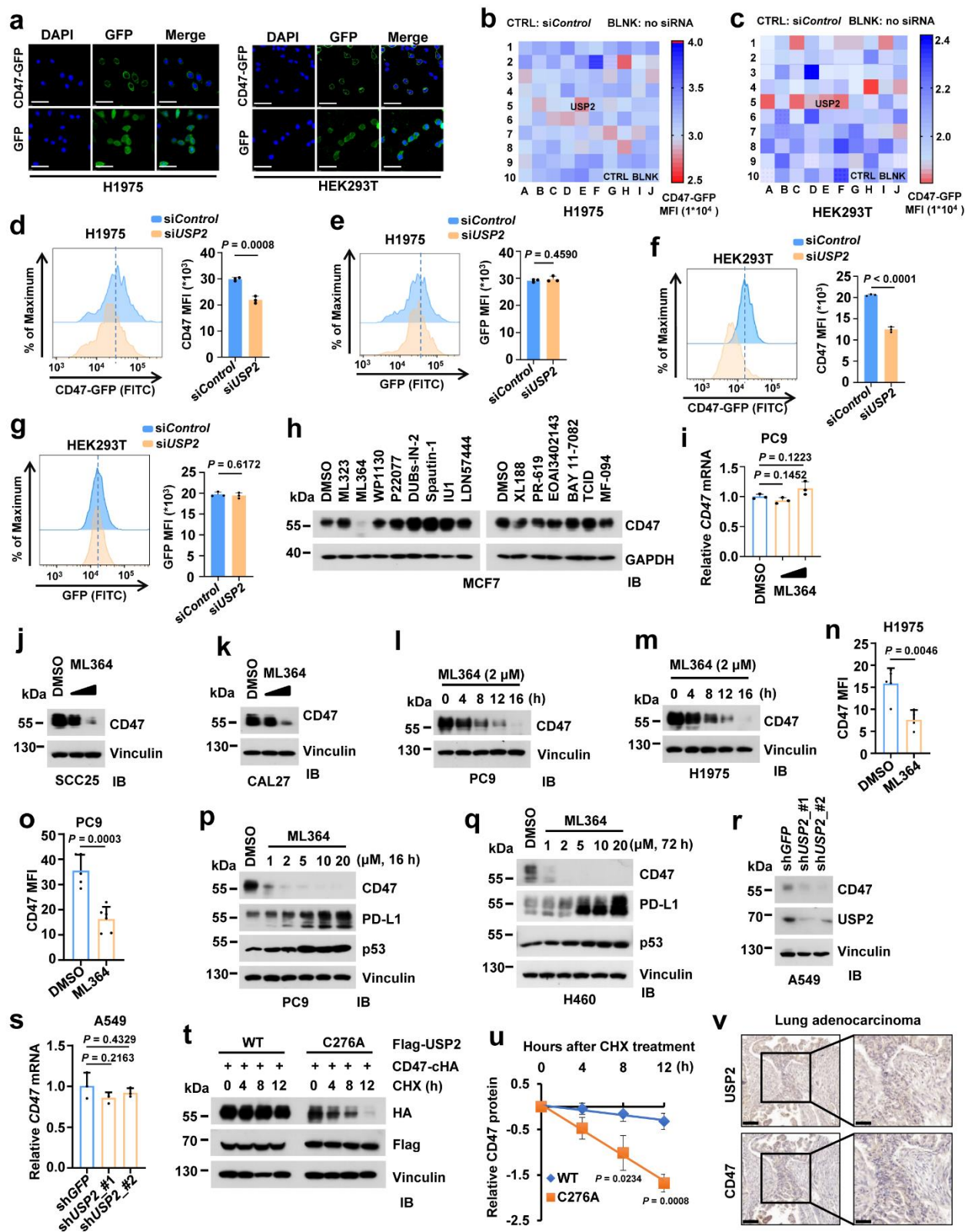
⁷Frontier Science Center for Immunology and Metabolism, Wuhan University, Wuhan, China

⁸Department of Oral and Maxillofacial Surgery, School and Hospital of Stomatology, Wuhan University, Wuhan 430079, China

⁹Wuhan Research Center for Infectious Diseases and Cancer, Chinese Academy of Medical Sciences, Wuhan, China

[#]These authors contributed equally

^{*}Corresponding author: geraldchan@whu.edu.cn (G.C.), yan.gong@whu.edu.cn (Y.G.), chxie_65@whu.edu.cn (C.X.), jinfang_zhang@whu.edu.cn (J.Z.)



Supplementary Fig. 1 The deubiquitinase USP2 positively regulates the CD47 protein abundance.

a, Immunofluorescence (IF) staining for CD47 in H1975 cells (left) and HEK293T cells (right) that stably expressing the pLenti-GFP empty vector or pLenti-CD47-GFP. Scale bar, 50 μ m.

b, c, Heatmaps illustrate the MFI of CD47-GFP signal through siRNA library-based deubiquitinase screening in H1975 (**b**) and HEK293T (**c**) cells expressing CD47-GFP. MFI values are presented in a heatmap, with colors ranging from blue to red. The MFI value following siUSP2 transfection are indicated in column E of row 5, while the siDUBs corresponding to the other entries in the heatmap are listed in the Source data file. MFI, mean fluorescence intensity. BLNK, no siRNA. CTRL, Control siRNA.

d-g, Cell surface CD47-GFP expressing in H1975 (**d**) and HEK293T (**f**) cells or GFP expressing in H1975 (**e**) and HEK293T (**g**) cells was analyzed by flow cytometry using the FITC channel.

h, Immunoblotting (IB) analysis of whole-cell lysates (WCL) for MCF7 cells treated with indicated deubiquitinase inhibitors (2.5 μ M) or dimethyl sulfoxide (DMSO) for 36 h.

i, The mRNA level of *CD47* in PC9 cells was measured using reverse transcription quantitative PCR (RT-qPCR).

j, k, IB analysis of WCL derived from SCC25 cells (**j**) or CAL27 cells (**k**) treated with ML364 (1 μ M and 2 μ M) for 16 h.

l, m, IB analysis of WCL derived from PC9 (**l**) cells or H1975 cells (**m**) treated with ML364 (2 μ M) at the indicated time points.

n, o, MFI of cell surface CD47 by IF staining in H1975 (**n**) and PC9 (**o**) cells from Fig. 1 (**f**) and (**g**) were quantified. H1975 cells (DMSO n = 5, ML364 n = 4); PC9 cells (DMSO n = 5, ML364 n = 6).

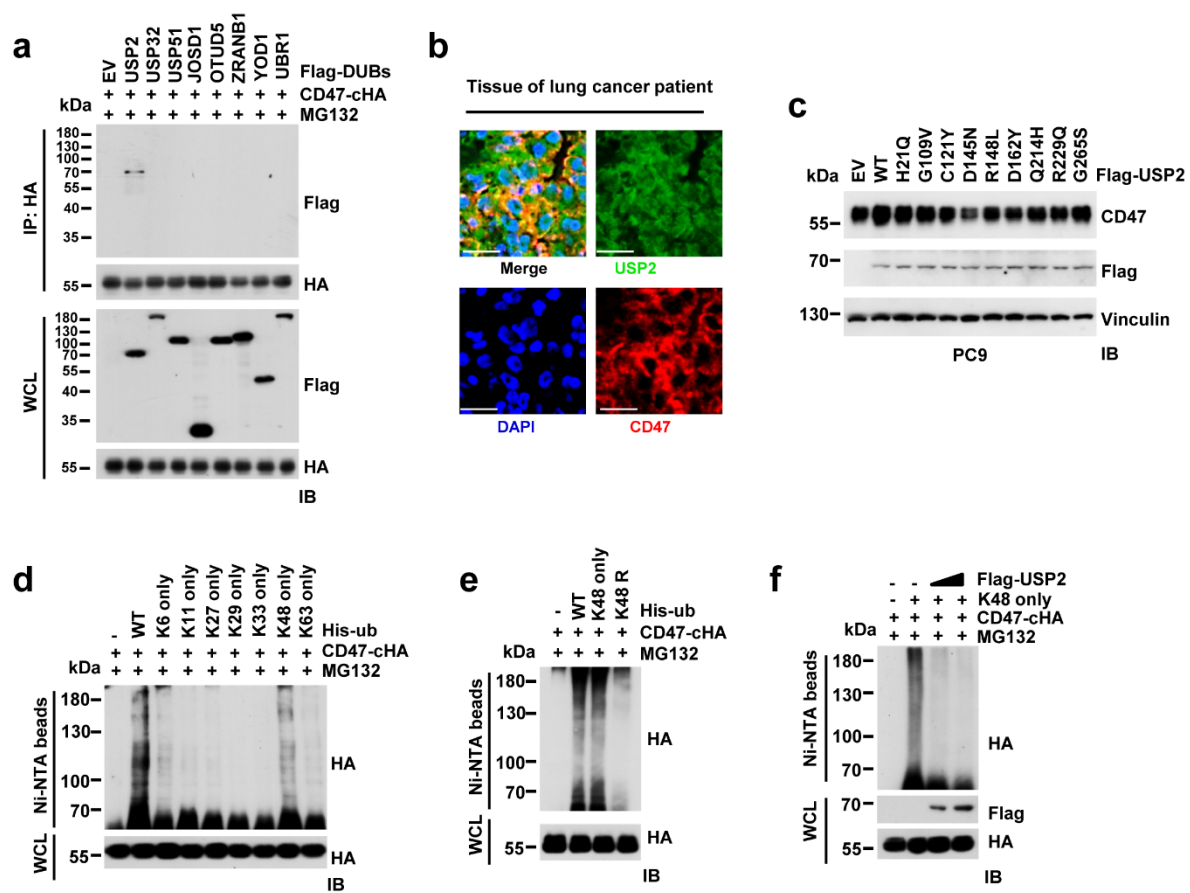
p, q, IB analysis of WCL derived from PC9 (**p**) cells or H460 cells (**q**) treated with ML364 (0, 1 μ M, 2 μ M, 5 μ M, 10 μ M and 20 μ M) at the indicated time points.

r, s, IB analysis of WCL derived from A549 cells stably expressing shUSP2 and shGFP (**r**). The mRNA level of *CD47* was measured using RT-qPCR (**s**).

t, u, IB analysis of WCL derived from HEK293T cells co-transfected with indicated constructs. Cells were treated with 200 μ g/ml cycloheximide (CHX) for the indicated time points (**t**). Quantification of CD47 protein band intensity was normalized to vinculin, then compared to the t = 0 time point (**u**).

v, Representative images of immunohistochemical (IHC) staining for CD47 and USP2 in human lung adenocarcinoma. Scale bar, left panels: 100 μ m; right panels: 50 μ m.

For **d-g, i, n, o, s**, and **u**, unpaired two-tailed Student's *t*-test. Data are shown as the mean $\pm$ SD. $P < 0.05$ was considered statistically significant. n = 3 biologically independent experiments for **d-m**, and **p-u**. Source data are provided as the Source Data file.



Supplementary Fig. 2 USP2 binds to CD47 and removes the K48-linked ubiquitination on CD47 in cells.

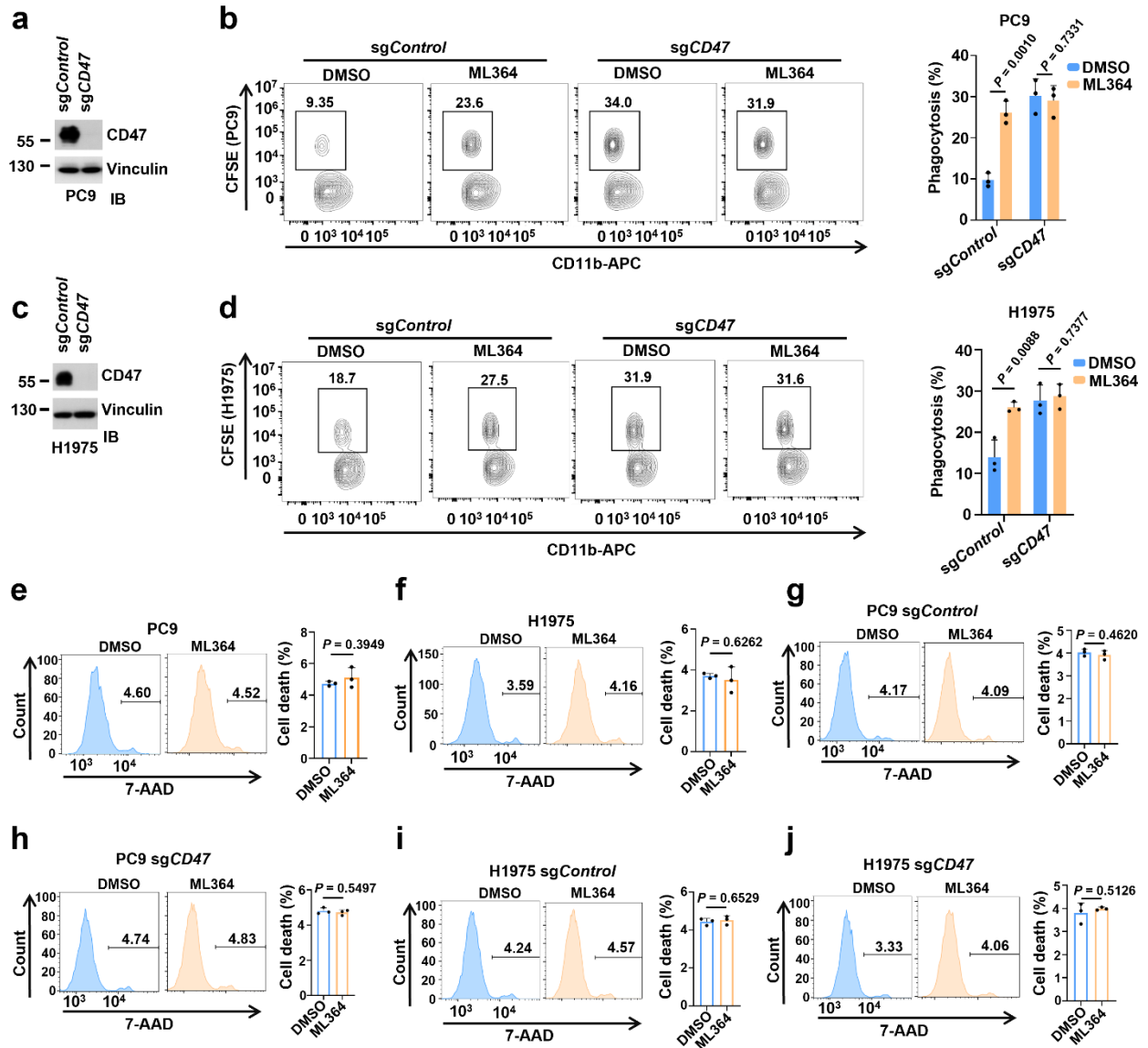
a, IB analysis of WCL for HEK293T cells and anti-HA immunoprecipitates (IPs). HEK293T cells were co-transfected with the indicated constructs and treated with 10 μ M MG132 for 12 hours before collection.

b, IF staining for USP2 in green, CD47 in red and DAPI in blue. Overlay cells in yellow elucidate the co-localization of USP2 and CD47 in human lung cancer tissue. Scale bar, 50 μ m.

c, IB analysis of WCL derived from PC9 cells transfected with indicated constructs.

d-f, IB analysis was performed on the WCL and Ni-NTA pull-down products from the *in vivo* ubiquitination assay, using a guanidine-HCl denaturing buffer. HEK293T cells were co-transfected with the indicated constructs and treated with 10 μ M MG132 for 12 hours before harvesting.

For **a**, **c-f**, $n = 3$ biologically independent experiments. Source data are provided as a Source Data file.



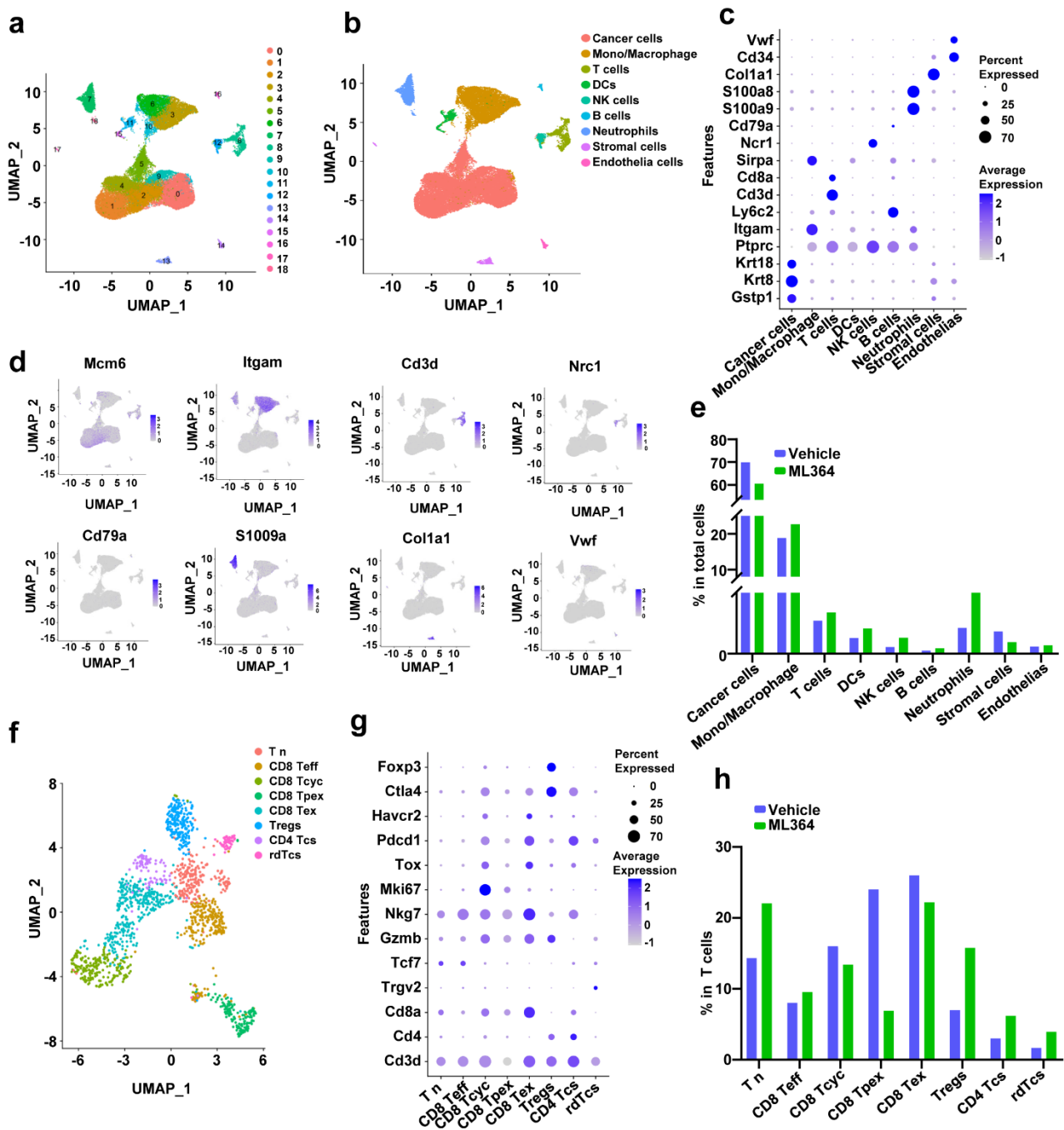
Supplementary Fig. 3 ML364-mediated USP2 inhibition enhances macrophage phagocytosis of lung cancer cells in a CD47-dependent manner.

a, c, IB analysis of WCL derived from PC9 cells (**a**) or H1975 cells (**c**) stably expressing *sgControl* or *sgCD47*.

b, d, Representative flow cytometry plots displaying THP-1 macrophage phagocytosis of PC9 (**b**) or H1975 cells (**d**) cells that stably expressing *sgControl* or *sgCD47* cells. Quantitative results for phagocytosis to show the percentage of CFSE⁺ PC9 cells (**b**) or H1975 cells (**d**) in CD11b⁺ cells (THP-1 macrophages).

e-j, Flow cytometry analysis of the cell death rates following ML364 (2 μ M) treatment for 16 h in PC9 cells (**e**); H1975 cells (**f**); PC9-*sgControl* cells (**g**); PC9-*sgCD47* cells (**h**); H1975-*sgControl* cells (**i**) and H1975-*sgCD47* cells (**j**). Cells were stained with 7-Amino-Actinomycin D (7-AAD).

For **b-j**, unpaired two-tailed Student's *t*-test, *n* = 3 independent biological replicates. Data are shown as the mean $\pm$ SD. *P* < 0.05 was considered statistically significant. *n* = 3 biologically independent experiments for (**a** and **c**). Source data are provided as the Source Data file.



Supplementary Fig. 4 Single-cell RNA sequencing (scRNA-seq) analysis shows that inhibition of USP2 with ML364 treatment modulates the tumor microenvironment *in vivo*.

a, b, Uniform Manifold Approximation and Projection (UMAP) of scRNA-seq data showing the 19 cell clusters (**a**) and 9 unique cell types (**b**) identified of subcutaneous LLC tumors derived from C57BL/6J mice treated with ML364 (5 mg/kg) or control vehicle for 12 days. Color coding indicates the different cell types. DCs, dendritic cells; NK cells, Natural killer cells.

c, Dot-plot showing the select representative gene markers enriched in cancer and infiltrating cells.

d, UMAP feature plots of cancer and infiltrating cells, displaying the selected marker gene expressed in the different cell clusters.

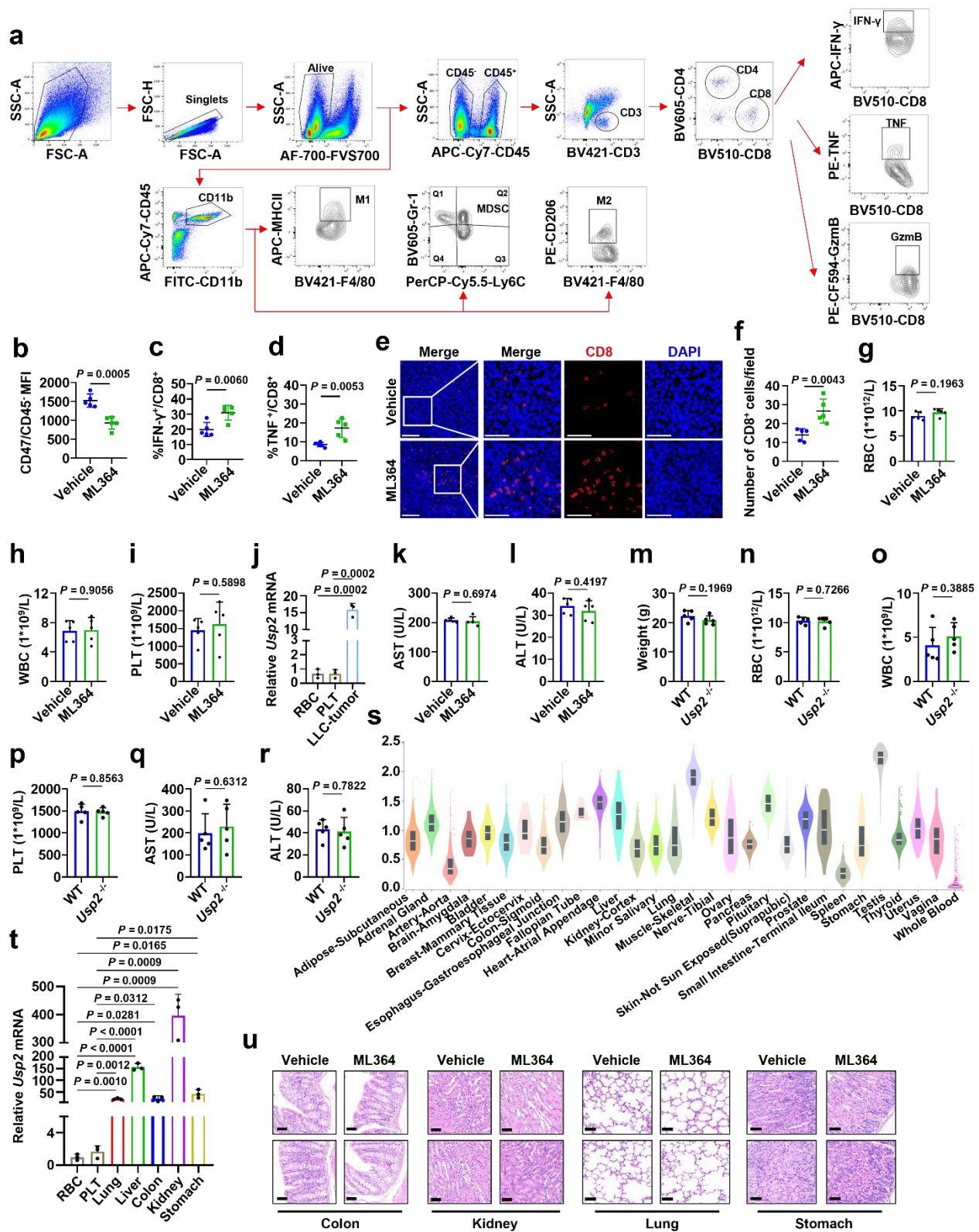
e, Comparative abundance of the cancer and infiltrating cells in LLC tumors treated with control vehicle (blue) versus ML364 (green) from the scRNA-seq data.

f, UMAP plot of scRNA-seq showing the subpopulations identified within the T cells. Color coding indicates the different cell types. T_n, naïve T cells; CD8 Teff, effector CD8 T cells; CD8 Tcyc, cycling CD8 T cells; CD8 Tpex, pre-exhausted CD8 T cells; CD8 Tex, exhausted CD8 T cells; Tregs, regulatory T cells; CD4 Tcs, CD4 T cells; rdTcs, gamma delta T cells.

g, Dot-plot showing the select representative gene markers enriched in T cells.

h, Comparative abundance of T cell subsets in LLC tumors treated with control vehicle (blue) versus ML364 (green) from the scRNA-seq data.

Source data are provided as the Source Data file.



Supplementary Fig. 5 ML364 treatment enhances cytotoxic CD8⁺ T cell infiltration and exhibits anti-tumor activity without significant toxicity.

a, The representative FACS gating strategies for analyzing tumor-infiltrating lymphocytes and myeloid cells. Cells were first gated by FSC-A and FSC-H to eliminate non-singlet cells and gated by FSC-A/AF-700-FVS700 to eliminate dead cells. This gating strategy is suitable for analyzing all tumor-infiltrating leukocytes.

b, Quantification of cellular surface CD47 on CD45⁺ cells in subcutaneous LLC tumors derived from mice with indicated treatments.

c d, Quantification of IFN- γ ⁺ cells (**c**) or TNF⁺ cells (**d**) represented as percentage of CD8⁺ T cells in subcutaneous LLC tumors derived from C57BL/6J mice treated with control vehicle or ML364 (5 mg/kg).

e, f, Representative IF staining images of CD8 (red) in subcutaneous LLC tumors with indicated treatment (**e**). Each point represents the average counts of positive cells within three high-power fields (**f**). Scale bars, 100 μ m (left); zoom scale bars, 50 μ m (right).

g-i, Complete blood cell counts (RBC, Red blood cell; WBC, White blood cell and PLT, Platelets) from mice treated with indicated treatments were measured.

j, The mRNA level of *Usp2* in RBC, PLT and LLC-tumor was measured using RT-qPCR.

k, l, Liver function was assessed by measuring the aspartate transaminase (AST) and alanine transaminase (ALT) from mice treated with control vehicle or ML364 (5 mg/kg).

m, The body weight of WT and *Usp2*^{-/-} mice at 8-week-old was measured.

n-p, RBC, WBC, and PLT from WT and *Usp2*^{-/-} mice at 8-week-old were measured.

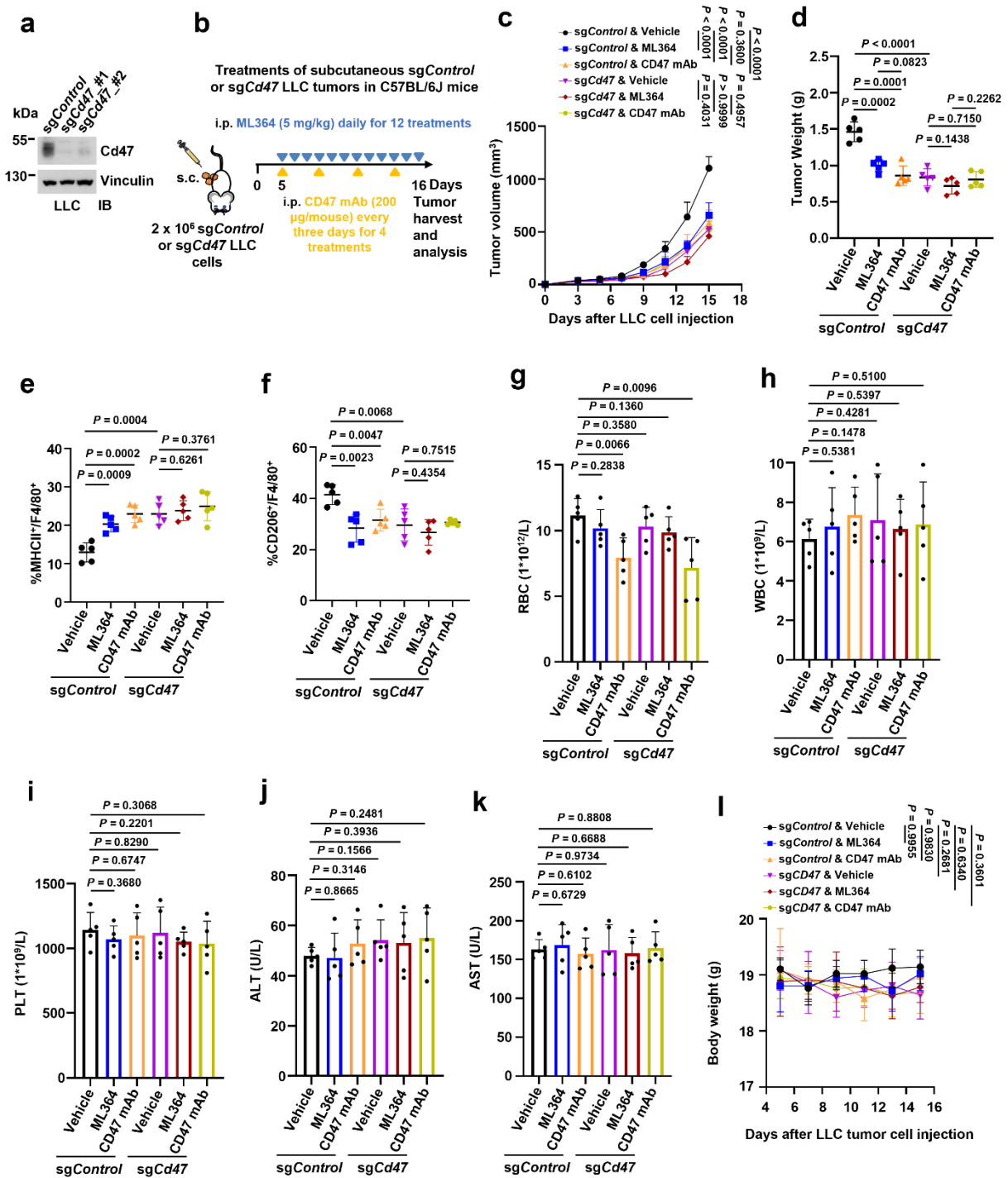
q, r, Liver function was assessed by measuring the AST and ALT from WT and *Usp2*^{-/-} mice at 8-week-old.

s, The *USP2* gene expression level in different human organs and tissues was displayed by utilizing GTEx portal (<https://www.gtexportal.org/home>) website. Expression values are shown in TPM (Transcripts Per Million), calculated from a gene model with isoforms collapsed to a single gene. Box plots are shown as median and 25th and 75th percentiles.

t, The mRNA level of *Usp2* in RBC, PLT, lung, liver, colon, kidney and stomach from WT mice at 8-week-old was measured using RT-qPCR.

u, Hematoxylin and Eosin staining (H&E) staining for the slices of the several organs (colon, kidney, lung, and stomach) from C57BL/6J mice following ML364 treatment. Scale bars, 50 μ m.

For **b-d**, **f-r**, and **t**, unpaired two-tailed Student's *t*-test. Data are shown as the mean $\pm$ SD. *n* = 5 mice per group (for **b-d**, **f-i**, and **k-r**). *n* = 3 biologically independent experiments for (**j** and **t**). Source data are provided as the Source Data file.



Supplementary Fig. 6 The anti-tumor effect of ML364 treatment is primarily dependent on the expression of CD47 in lung cancer cells.

a, IB analysis of WCL derived from LLC cells stably expressing *sgControl* or *sgCd47*, respectively.

b, A schematic treatment plan for immunocompetent C57BL/6J mice bearing *sgControl*- or *sgCd47*(#1)-LLC tumors. Mice were subcutaneously implanted with 2×10^6 *sgControl* or *sgCd47* LLC cells and treated with a control vehicle, USP2 inhibitor (ML364, 5 mg/kg), or CD47 mAb (200 µg/mouse), respectively. i.p., intraperitoneal; s.c., subcutaneous.

c, d, Tumor volume for each treatment group was measured using calipers, and the tumor growth curve was plotted (**c**). The weight of LLC tumors was measured at the endpoint (**d**).

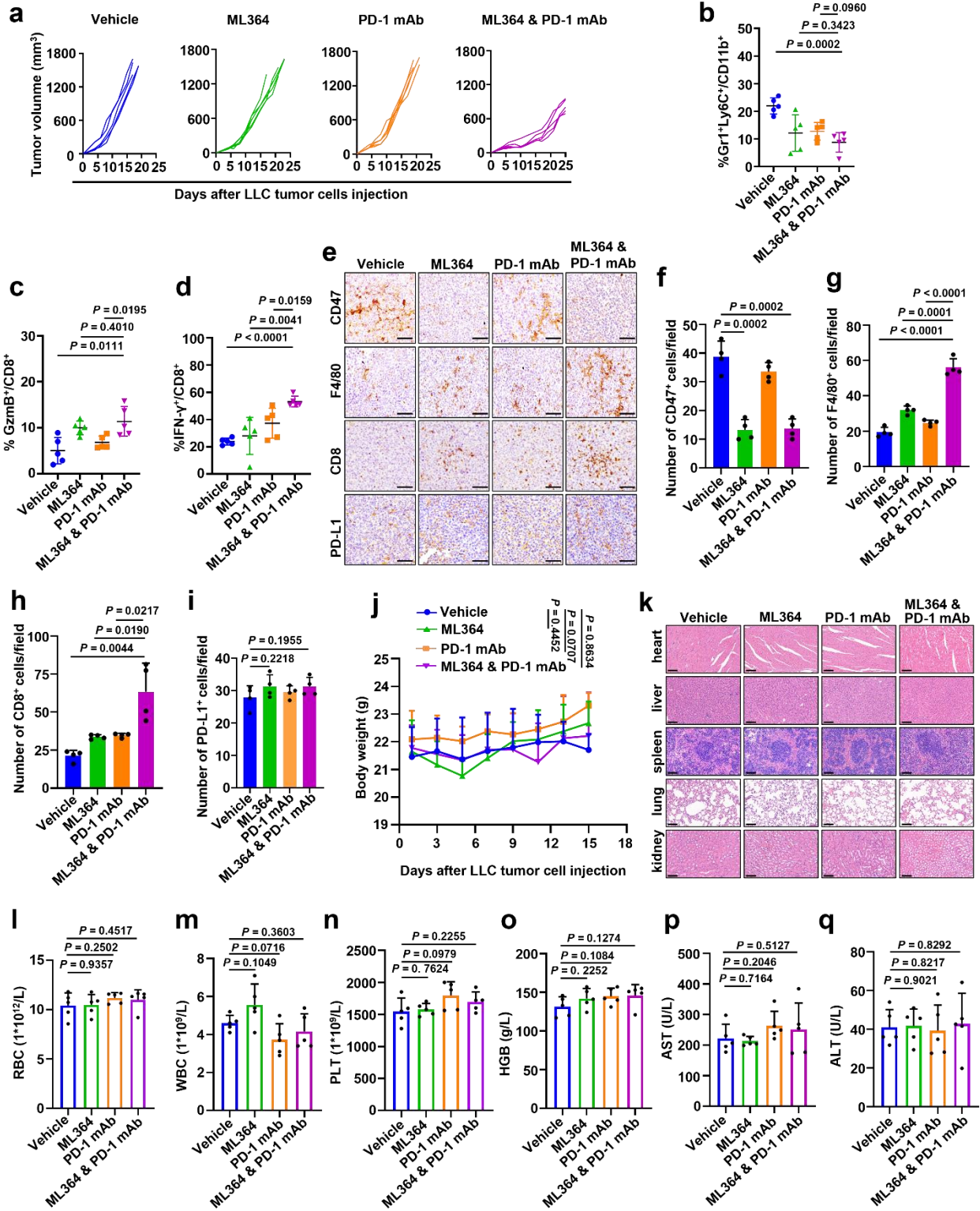
e, f, Quantification of MHCII⁺ (M1 macrophage) cells (**e**) or CD206⁺ (M2 macrophage) cells (**f**) represented as percentage of F4/80⁺ macrophages in subcutaneous LLC tumors derived from C57BL/6J mice treated with indicated reagents.

g-i, RBC, WBC and PLT from C57BL/6J mice with indicated treatments were measured.

j, k, Liver function was assessed by measuring AST and ALT levels in C57BL/6J mice treated with the indicated treatments.

l, The body weight of C57BL/6J mice was monitored over time during the indicated treatment.

For **d-k**, unpaired two-tailed Student's *t*-test. Two-way ANOVA for (**c**) and (**l**). Data are shown as the mean ± SD. *P* < 0.05 was considered statistically significant. n = 5 mice per group for (**c-l**). n = 3 biologically independent experiments for **a**. Source data are provided as the Source Data file.



Supplementary Fig. 7 The combination of ML364 with anti-PD-1 therapy significantly suppresses the subcutaneous LLC tumor growth without apparent toxicity in mice.

a, Tumor volume for different treatment groups was measured with caliper and the tumor growth curve was plotted.

b-d, Quantification of Gr1⁺Ly6C⁺ cells (myeloid derived suppressor cells, MDSCs) represented as percentage of CD11b⁺ monocytes (**b**), GzmB⁺ cells (**c**) or IFN- γ ⁺ cells (**d**) represented as percentage of CD8⁺ T cells in subcutaneous LLC tumors derived from C57BL/6J mice treated with indicated reagents.

e, IHC staining for CD47, F4/80, CD8 and PD-L1 in LLC tumors from C57BL/6J mice treated with indicated reagents. Scale bar, 50 μ m.

f-i, Quantification for CD47 (**f**), F4/80 (**g**), CD8 (**h**) and PD-L1 (**i**) positive cells. Each point represents the average counts of positive cells within three high-power fields. n = 4 mice per group.

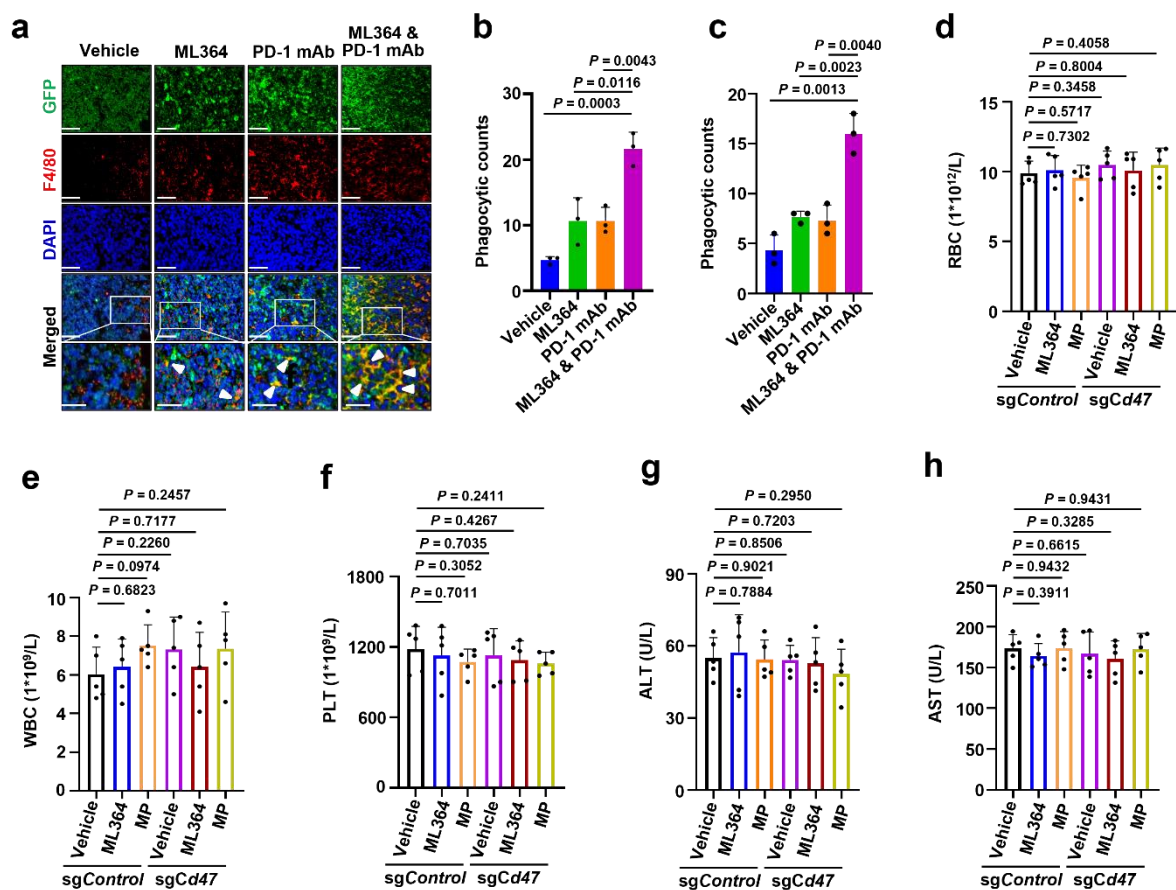
j, Time-course of body weight of C57BL/6J mice during the indicated treatment was monitored.

k, H&E staining for the slices of the several organs (heart, liver, spleen, lung, and kidney) from C57BL/6J mice with indicated treatments. Scale bar, 100 μ m.

l-o, RBC, WBC, PLT and HGB (Hemoglobin) from C57BL/6J mice with indicated treatments were measured.

p, q, Liver function was assessed by measuring the AST and ALT from C57BL/6J mice with indicated treatments.

For **b-d**, **f-i**, and **l-q**, unpaired two-tailed Student's *t*-test. Two-way ANOVA for (**j**). Data are shown as the mean $\pm$ SD. $P < 0.05$ was considered statistically significant. n = 5 mice per group (for **a-d**, **j**, and **l-q**). Source data are provided as the Source Data file.



Supplementary Fig. 8 ML364 alone or combined with anti-PD-1 shows no significant toxicity in mice bearing LLC tumors.

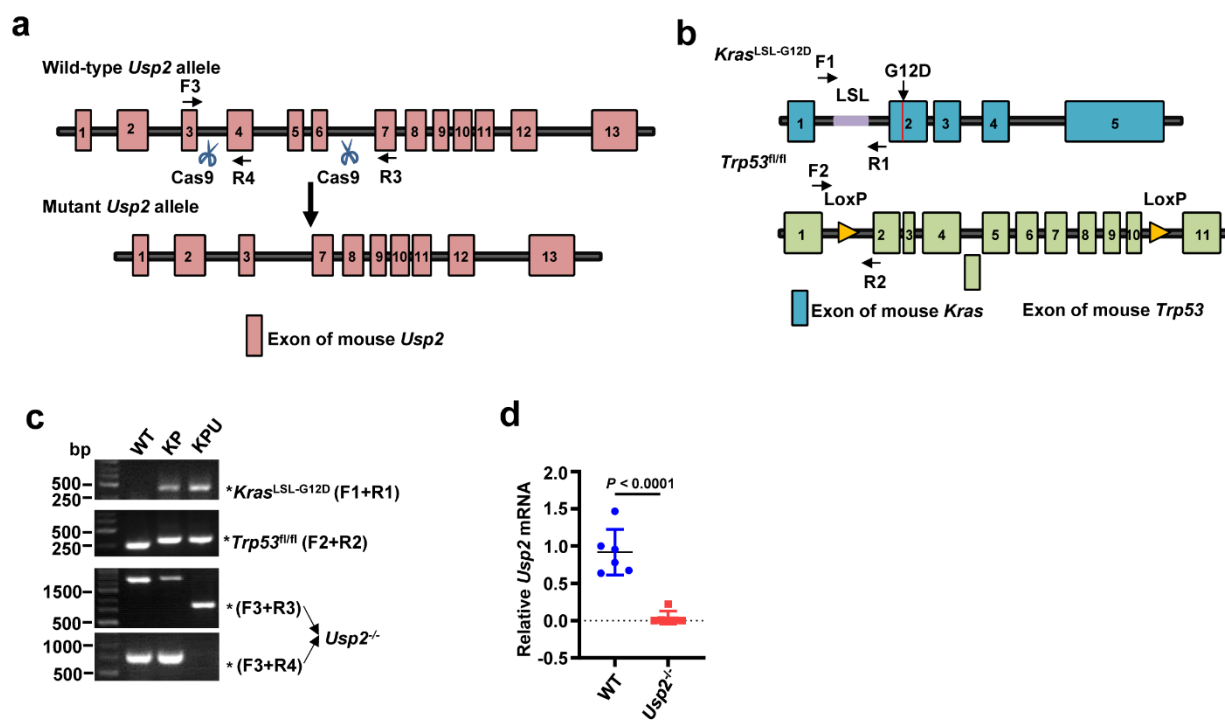
a, Representative images of IF staining showing tumor cells (GFP, green), macrophages (F4/80, red), and nuclei (DAPI, blue). The yellow signal (indicated by arrows) highlights the colocalization of GFP (green) and F4/80 (red), indicating macrophage-mediated phagocytosis of tumor cells. Each data point represents the average count of yellow cells from three high-power fields. Sections were scanned at 20× magnification with the eyepiece as a supplementary figure for (Fig. 4m). 100 μm (upper panels); zoom scale bars, 50 μm (lower panels).

b, c, Quantification of yellow colocalized cells was carried out in high-power fields. **(b)** quantification for **(a)** images; **(c)** quantification for (Fig. 4m) images. Each point represents the average counts of positive cells within three high-power fields. n = 3 mice per group.

d-f, RBC, WBC, and PLT counts were measured in C57BL/6J mice bearing *sgControl*- or *sgCd47*-LLC tumors following the indicated treatments. MP, ML364 & PD-1 mAb combined therapy.

g, h, Liver function was evaluated by measuring AST and ALT levels in C57BL/6J mice bearing *sgControl*- or *sgCd47*-LLC tumors, with the indicated treatments.

For **b-h**, unpaired two-tailed Student's *t*-test. Data are shown as the mean ± SD. n=5 mice per group for **(d-h)**. *P* < 0.05 was considered statistically significant. Source data are provided as the Source Data file.



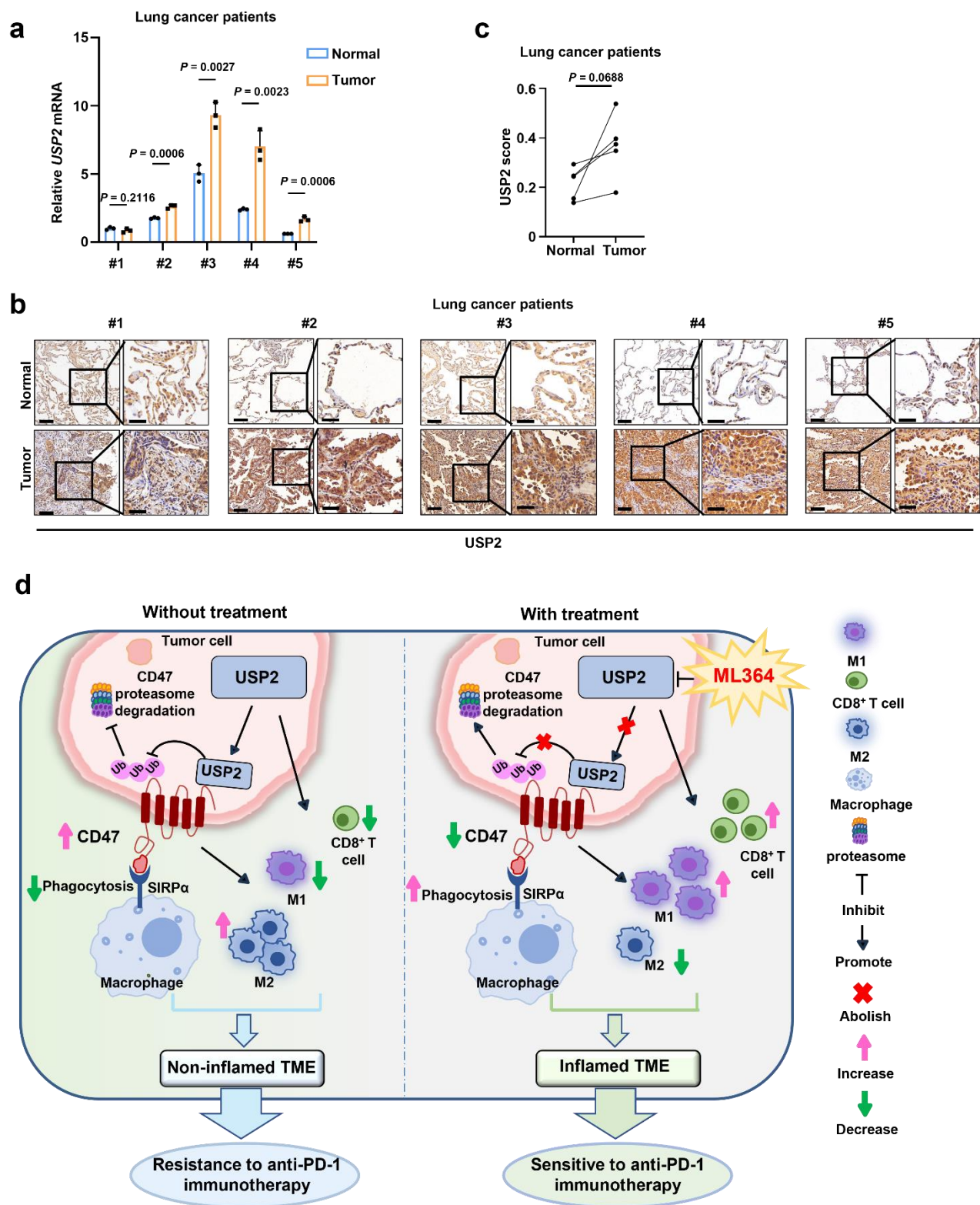
Supplementary Fig. 9 Construction strategy of KP and KPU mice.

a, b, The schematic illustration of generating *Kras*^{LSL-G12D/+}; *Trp53*^{fl/fl}; *Usp2*^{-/-} (KPU) mice strategies by crossing *Kras*^{LSL-G12D/+}; *Trp53*^{fl/fl} (KP) with *Usp2*^{-/-} mice.

c, The genotypes of WT, KP and KPU mice were visualized by gel electrophoresis.

d, The mRNA level of *Usp2* from lung tissues of *Usp2*^{-/-} mice was quantified by RT-qPCR. n = 6 mice per group.

For **(d)**, unpaired two-tailed Student's *t*-test. Data are shown as the mean $\pm$ SD. *P* < 0.05 was considered statistically significant. Source data are provided as the Source Data file.



Supplementary Fig. 10 Assessment of USP2 expression at the transcript and protein levels in lung cancer patient tissues.

a. The mRNA level of *USP2* in tumor and adjacent non-tumor tissues from lung cancer patients was measured by RT-qPCR.

b, c, Representative images of IHC staining for USP2 in tumor and adjacent non-tumor tissues from lung cancer patients (**b**). Scale bar, left panels: 100 μm ; right panels: 50 μm . Quantification of USP2 IHC staining intensities were quantified as AOD (**c**). $n = 5$.

d, A proposed framework suggests that USP2 inhibition sensitizes tumors to anti-PD-1 therapy. Elevated USP2 expression upregulates CD47 protein levels, thereby suppressing macrophage-mediated phagocytosis of tumor cells, reducing M1 macrophage populations, and promoting M2 polarization. It also reduces CD8⁺ T cell infiltration, contributing to the formation of a non-inflamed tumor microenvironment (TME) that promotes resistance to anti-PD-1 therapy. Conversely, USP2 inhibition by ML364 reduces CD47 levels, enhancing macrophage phagocytosis and increasing M1 macrophages and CD8⁺ T cells infiltration. This shift remodels the TME into an inflamed state, making tumor cells more responsive to anti-PD-1 immunotherapy. Ub: ubiquitin.

For **a**, unpaired two-tailed Student's *t*-test, for **c**, paired two-tailed Student's *t*-test. Data are shown as the mean $\pm$ SD. $P < 0.05$ was considered statistically significant. $n = 3$ biologically independent experiments for **a**. Source data are provided as the Source Data file.

Supplementary Table 1. Features of lung cancer patients’ specimen used in this study for Fig. 8f-i.

Clinical characteristics of lung cancer patients undergoing immunotherapy		
Patient ID	Gender	Age range
1	Male	50-69
2	Male	
3	Male	
4	Male	
5	Male	
6	Male	
7	Female	
8	Female	
9	Male	
10	Male	
11	Male	
12	Male	

Supplementary Table 2. Features of oral cancer patients’ specimen used in this study for Fig. 8j-m.

Clinical characteristics of OSCC patients undergoing immunotherapy		
Patient ID	Gender	Age range
2	Male	30-69
3	Male	
4	Male	
5	Female	
6	Female	
8	Male	
9	Male	
10	Male	
11	Male	
13	Male	
15	Male	
16	Male	
17	Male	
18	Male	
19	Female	
20	Male	
21	Male	
22	Female	
23	Male	
24	Male	
26	Male	
27	Female	
28	Male	
29	Male	
32	Male	
34	Female	
35	Male	
36	Male	
37	Male	
38	Male	
40	Male	
47	Female	
48	Male	
49	Male	
52	Male	
53	Female	
56	Male	
58	Male	
59	Male	
60	Male	
63	Male	
64	Male	
65	Male	
66	Male	

Supplementary Table 3. Features of lung cancer patients’ specimen used in this study for supplementary Fig. 10a-c.

Clinical characteristics of lung cancer patients		
Patient ID	Gender	Age range
1	Male	50-79
2	Male	
3	Female	
4	Male	
5	Male	