

Supplementary information

Inhibition of MBTPS1 Enhances Antitumor Immunity and Potentiates Anti-PD-1 Immunotherapy

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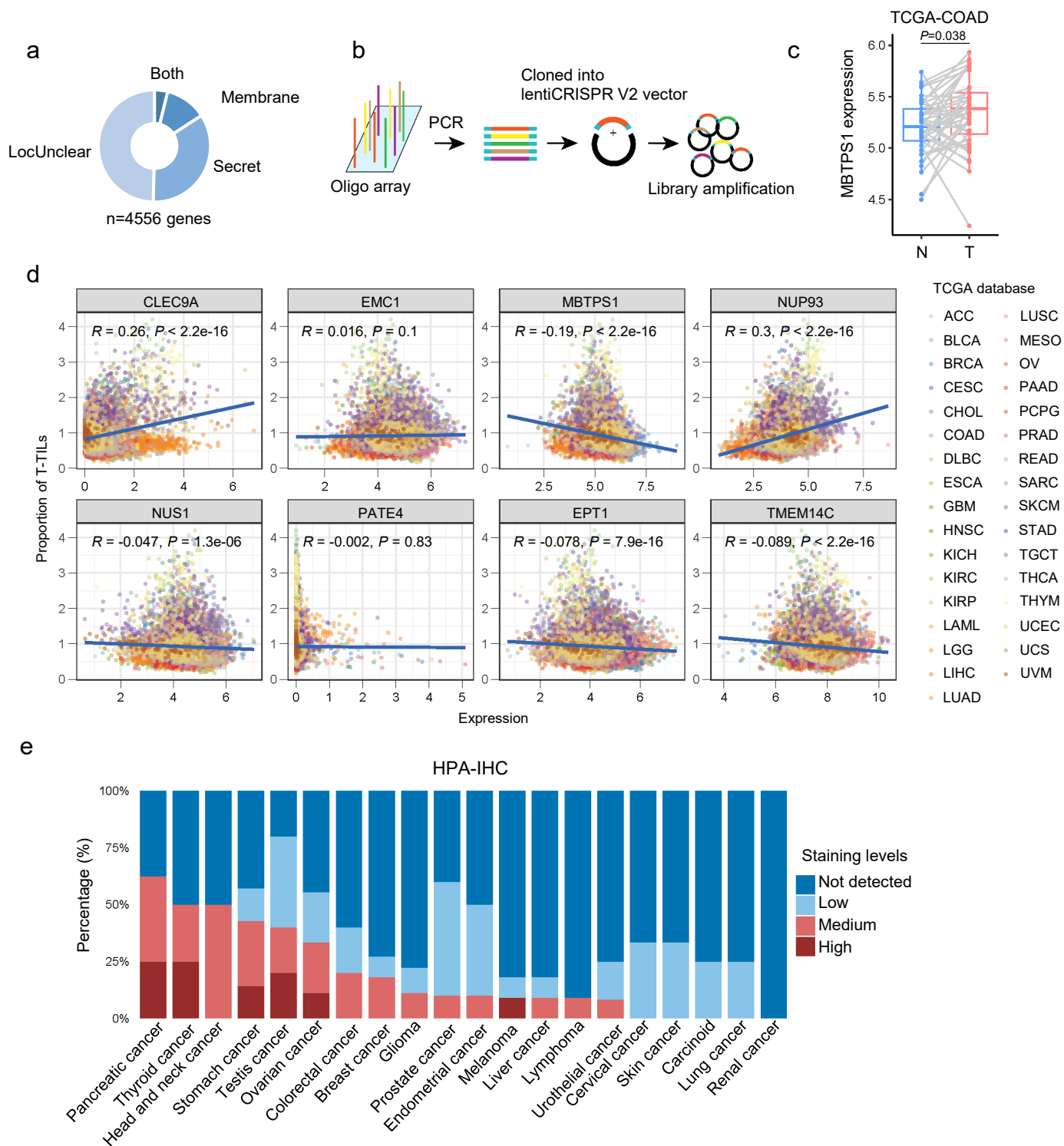
Supplementary Figure 6: MBTPS1 suppresses the production of chemokines by reducing the expression of STAT1.

Supplementary Figure 7: MBTPS1 disrupts the stability of STAT1 by competing with USP13 for binding to STAT1.

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Supplementary Figures 10 and 11: Gating strategy of flow cytometry assay.



Supplementary Fig. 1 An *in vivo* CRISPR screen focused on membrane and secreted proteins identifies MBTPS1 as an immunotherapy target.

a Pie chart showing the composition ratio of genes in the CRISPR-Cas9 knockout library (membrane proteins account for 12%; secreted proteins account for 34%; membrane/secreted proteins account for 4%; proteins with transmembrane sequence but unclear localization account for 50%).

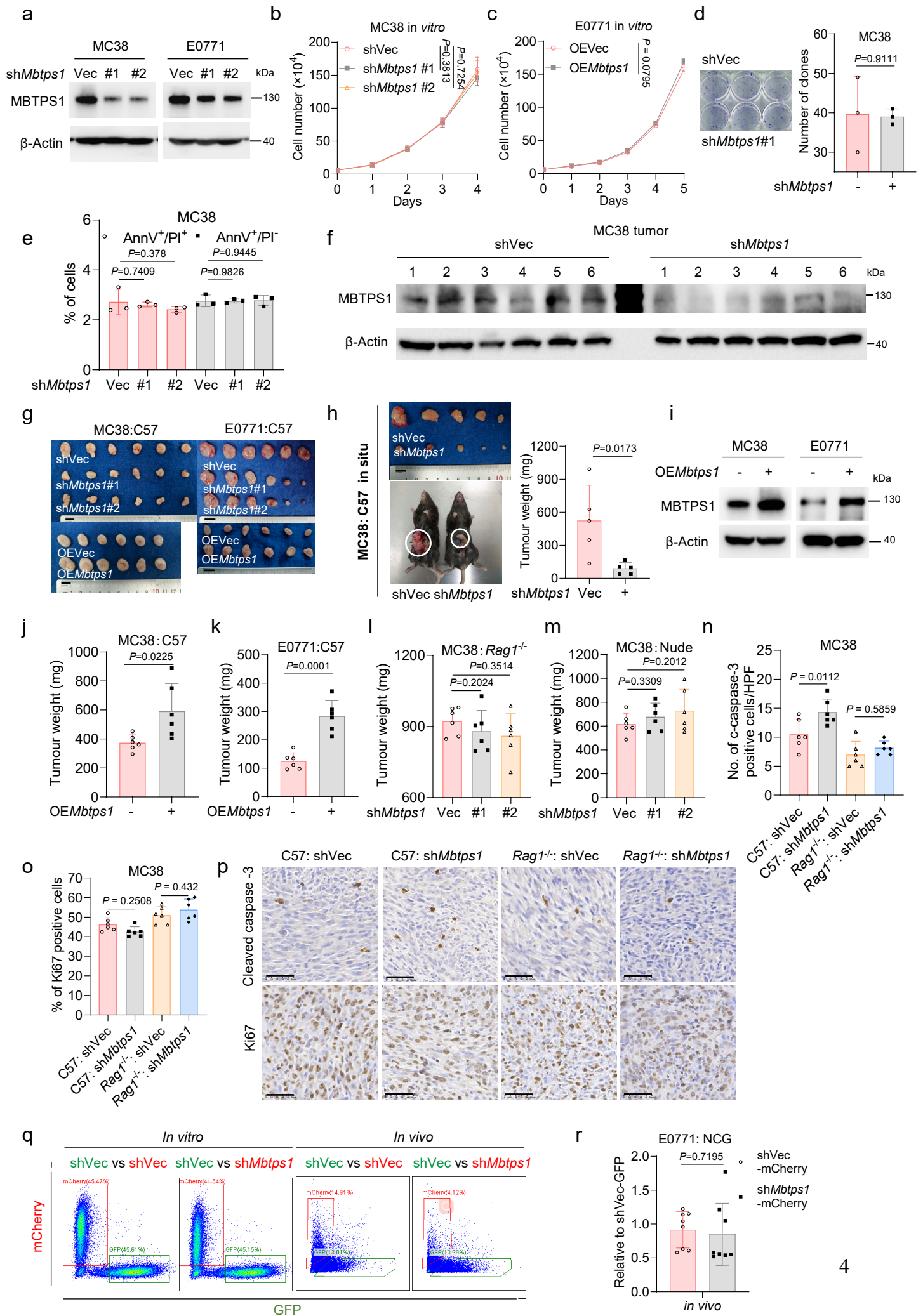
b Construction of CRISPR library based on membrane protein and secreted protein genes.

c The expression distribution of MBTPS1 in paired tumor tissues and normal tissues in TCGA-COAD dataset.

d Pearson correlation between T-TILs proportion and the expression of the top 10 genes across 33 cancer types from TCGA.

e Immunohistochemistry (IHC) staining data for MBTPS1 from Human Protein Atlas (HPA).

P values were determined by Pairwise Wilcox test for **c** and Spearman correlation analysis for **d**.



Supplementary Fig. 2 Loss of MBTPS1 inhibits tumor growth in immunocompetent mice.

a Immunoblotting (IB) detection of MBTPS1 in shVec and sh*Mbtps1* cells. β -Actin was included as a loading control.

b, c Growth curves of shVec/sh*Mbtps1* MC38 cells (**b**) and OEVec/OE*Mbtps1* E0771 cells (**c**) *in vitro*.

d Tumor image and statistical analysis of colony formation of shVec and sh*Mbtps1* MC38 cells.

e Quantification analysis of Annexin V/PI staining evaluating the percentages of early apoptotic cells (Annexin V⁺/PI⁻) and late apoptotic cells (Annexin V⁺/PI⁺).

f IB detection of MBTPS1 in MC38 tumors with *Mbtps1* knockdown. β -Actin was included as a loading control.

g Tumor images of shVec, sh*Mbtps1*, OEVec and OE*Mbtps1* MC38 or E0771 tumors in female C57 mice (6-8 weeks old). Scale bar, 1 cm.

h Tumor image and weights of shVec or sh*Mbtps1* tumors orthotopically transplanted into cecum of C57 mice. Scale bar, 1 cm.

i IB detection of MBTPS1 in OEVec and OE*Mbtps1* cells. β -Actin was included as a loading control.

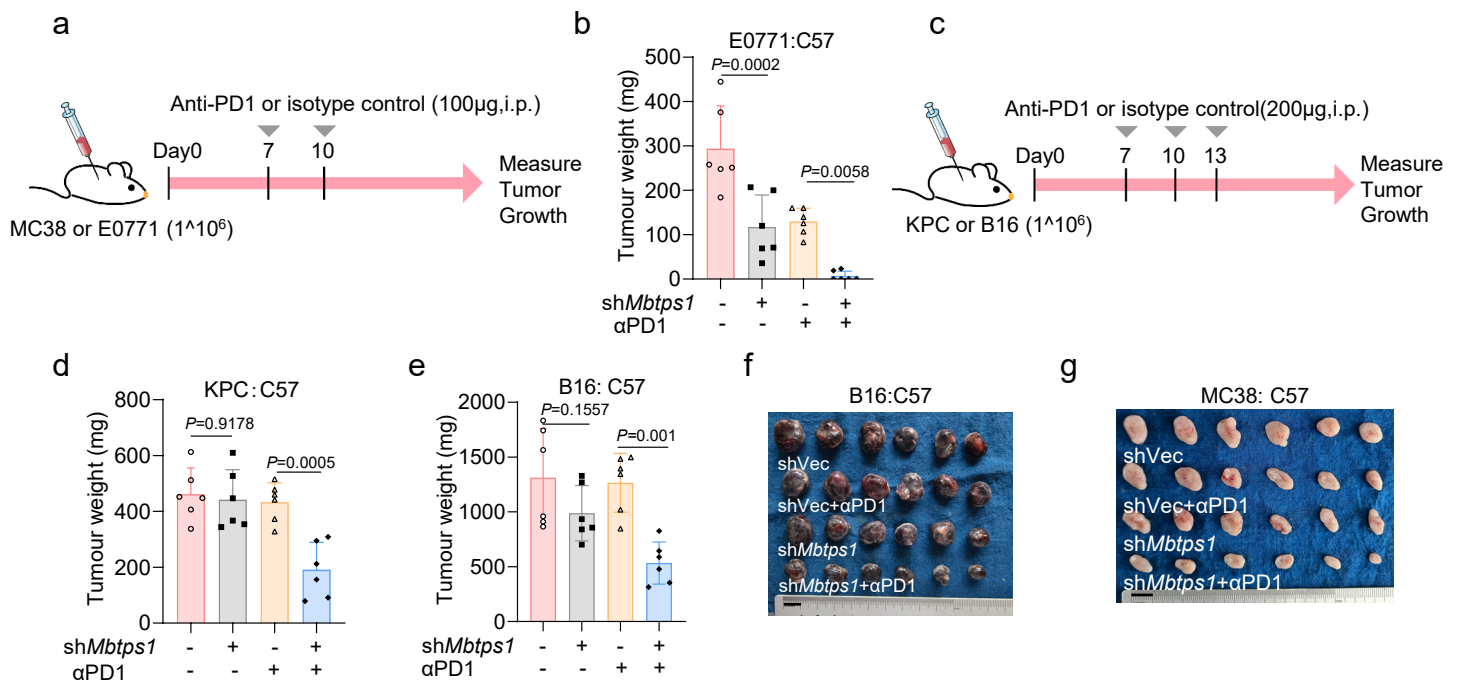
j, k Weights of OEVec and OE*Mbtps1* MC38 (**j**) or E0771 (**k**) tumors in C57 mice.

l, m Weights of shVec or sh*Mbtps1* MC38 tumors in female *Rag1*^{-/-} mice (5-6 weeks old) (**l**) and in female BALB/c Nude mice (5-6 weeks old) (**m**).

n, p Percentages of cleaved caspase-3 positive cells (**n**) and Ki67 positive cells (**o**) and representative IHC images (**p**) in MC38 tumors from *Rag1*^{-/-} and C57 mice analyzed by IHC staining. Scale bar: 50 μ m.

q Representative images of the ratio of shVec-mCherry to shVec-GFP and shVec-mCherry to sh*Mbtps1*-GFP cells cultured *in vitro* or in C57 mice.

r Changes in the ratio of shVec-mCherry to shVec-GFP and shVec-mCherry to sh*Mbtps1*-GFP E0771 in female NCG mice (5-6 weeks old). *n* = 3 biologically independent samples per group, representative of three independent experiments with similar results in **b-e**; *n* = 6 mice per group in **f, g** and **j-o**; *n* = 5 mice per group in **h**; *n* = 8 mice per group in **r**. IB experiments in **a** and **i** were repeated three times with similar results using biologically independent samples. Data are presented as means $\pm$ SDs. *P* values were determined by two-tailed unpaired Student's *t*-test in **b-e, g, h, j-m** and **r** and one-way ANOVA with Sidak's multiple comparisons test in **n** and **o**.



Supplementary Fig. 3 Loss of MBTPS1 sensitizes tumors to immunotherapy.

a The administration strategy for PD-1 antibody in MC38 and E0771 mouse models.

b Weight analysis of shVec and sh*Mbtps1* E0771 tumors in female C57 mice (6-8 weeks old) treated with α -PD-1 or isotype control.

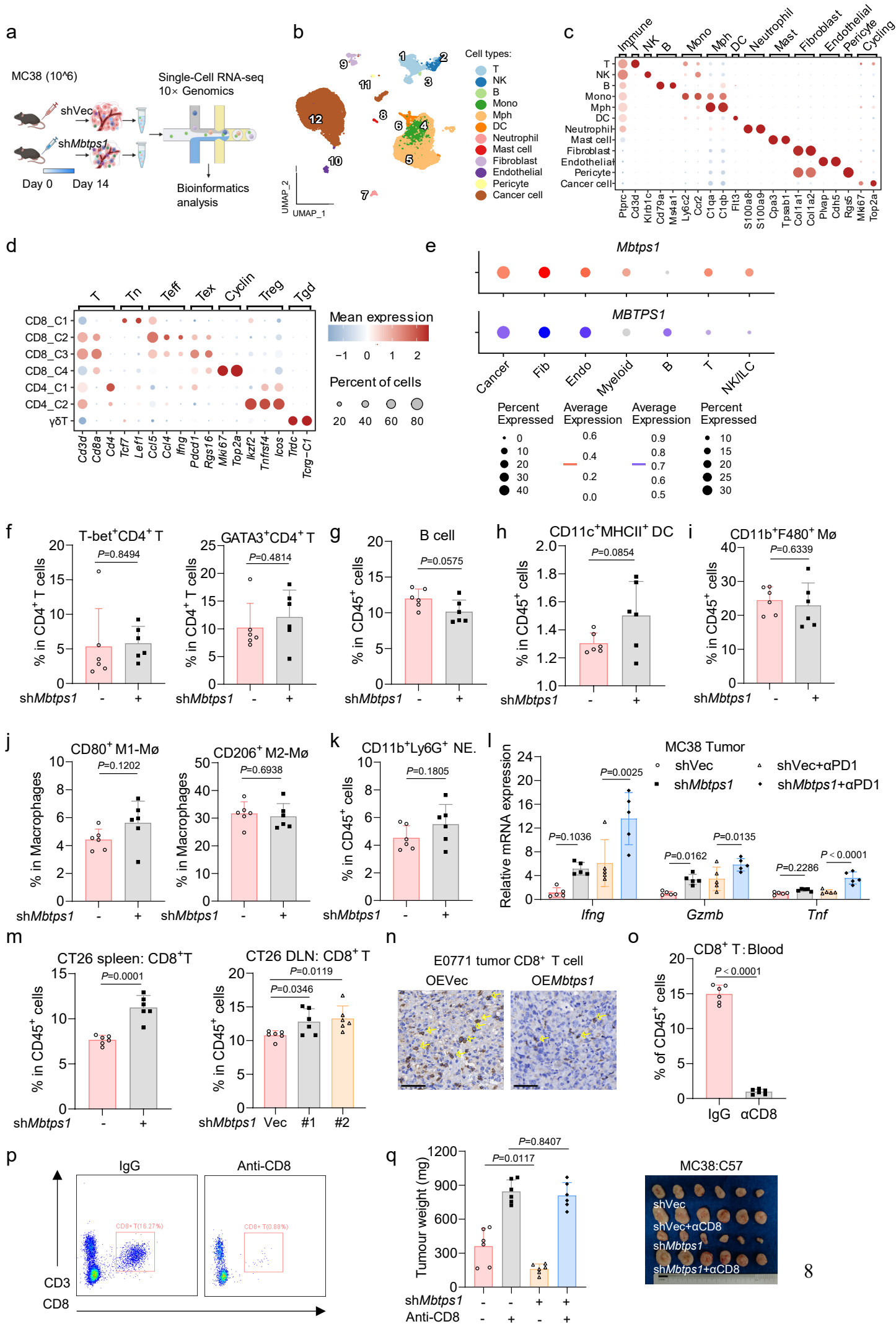
c The administration strategy for PD-1 antibody in KPC and B16 mouse models.

d, e Weight analysis of shVec and sh*Mbtps1* KPC tumors (d) or B16 tumors (e) in C57 mice treated with α -PD-1 or isotype control.

f Tumor image of **e**. Scale bar, 1 cm.

g Tumor image of C57 mice after implantation of shVec or sh*Mbtps1* MC38 cells, followed by intraperitoneal (i.p.) injection of α -PD-1 (2.5 mg/kg) or isotype control. Scale bar, 1 cm.

n = 6 mice per group in **b**, **d** and **e**. Data are presented as means $\pm$ SDs. *P* values were determined one-way ANOVA with Sidak's multiple comparisons test in **b**, **d** and **e**.



Supplementary Fig. 4 MBTPS1 depletion increases CD8⁺ T cell infiltration into the TME.

a Schematic diagram of single-cell RNA-seq performed on mouse tumors.

b UMAP projection of 21,444 cells isolated from tumor tissues isolated from C57 wild-type mice bearing either *Mbtps1-knockdown* or control MC38 cells.

c, d Dot plots of relative expression levels and the percentage of cells expressing marker genes among all cells (c) and specifically within lymphocytes (d).

e Upper: expression levels of *Mbtps1* in various cell types from the scRNA-seq data in supplementary figure 4a. Lower: expression level of *MBTPS1* in different cell types in patients with CRC analyzed by scRNA-seq data¹⁷.

f-k Percentages of T-bet⁺CD4⁺ T cells, GATA3⁺CD4⁺ T cells (f), B cells (g), DC cells (h), macrophages (i), M1-type macrophages, M2-type macrophages (j) and neutrophils (k) in shVec and sh*Mbtps1* MC38 tumors analyzed by flow cytometry.

l qPCR analysis of *Ifng*, *Gzmb* and *Tnf* mRNA expression in tumors from the indicated groups.

m Flow cytometry analysis of the percentages of CD8⁺ T cells in spleens or tumor-draining lymph nodes of C57 mice transplanted with shVec or sh*Mbtps1* MC38 tumors.

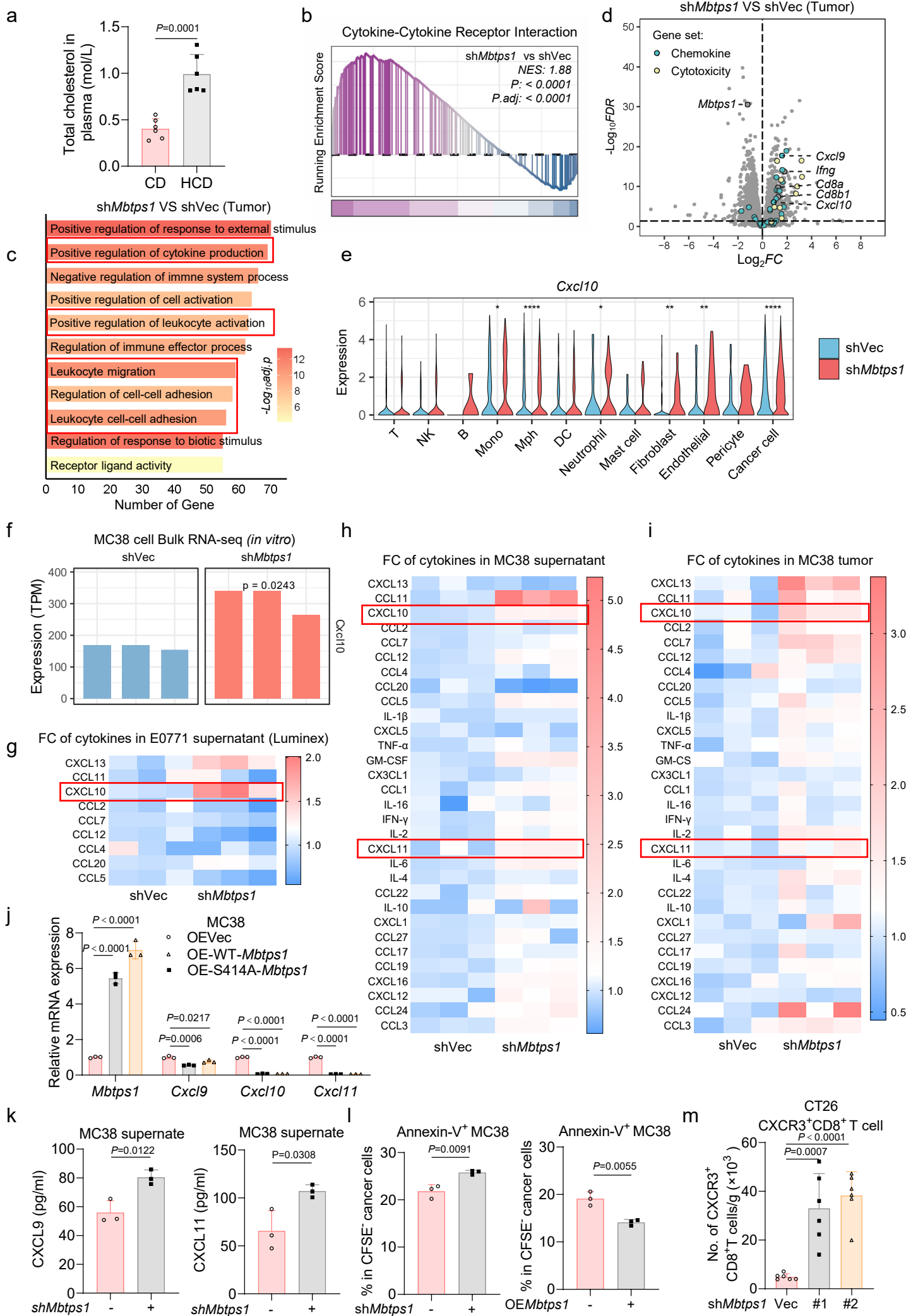
n Representative images of IHC stained CD8 in OEVec and OEM*Mbtps1* E0771 tumors. The yellow arrows indicate the location of CD8-positive cells stained by IHC. Scale bar, 50 μ m.

o Analysis of the flow cytometry assay conducted on the peripheral blood of C57 mice treated with CD8 neutralization antibody.

p Representative images demonstrating the effectiveness of the CD8 neutralization antibody.

q Weight analysis and tumor image and of shVec and sh*Mbtps1* MC38 tumors in C57 mice treated with CD8 neutralization antibody or isotype control. Scale bar, 1 cm.

n = 6 mice per group in **f-k**, **m**, **o** and **q**; n = 5 mice per group in **l**. Data are presented as means $\pm$ SDs. Two-tailed unpaired Student's *t* test for **f-k**, **m** and **o**; One-way ANOVA with Sidak's multiple comparisons test for **l** and **q**.



Supplementary Fig. 5 MBTPS1 inhibits antitumor immunity by regulating chemokines with chemotactic effects on CD8⁺ T cells rather than through its classical functions.

a The total cholesterol concentration in the plasma of female C57 mice (6-8 weeks old) fed with HCD or CD diet.

b GSEA enrichment plot of the cytokine-cytokine receptor interaction signaling pathway in MC38 tumors, indicating significant enrichment in sh*Mbtps1* tumors.

c Gene ontology analysis of biological processes using up-regulated genes in sh*Mbtps1* versus shVec tumor tissues. Red frames highlight the pathways related to cytokine production, as well as lymphocyte adhesion, migration, and activation.

d Volcano plot showing differentially expressed genes between sh*Mbtps1* and shVec MC38 tumor tissues, with genes encoding chemokines and cytotoxic particles highlighted in color.

e Violin plots showing the *Cxcl10* mRNA expression in various cell types analyzed using bulk scRNA-seq data.

f Bar graph showing the *Cxcl10* mRNA expression in MC38 cells analyzed using bulk RNA-seq data.

g-i Heatmaps displaying the fold change of a panel of 31 chemokines in the culture supernatant of E0771 cells (f), MC38 cells (g), and in MC38 tumors (h).

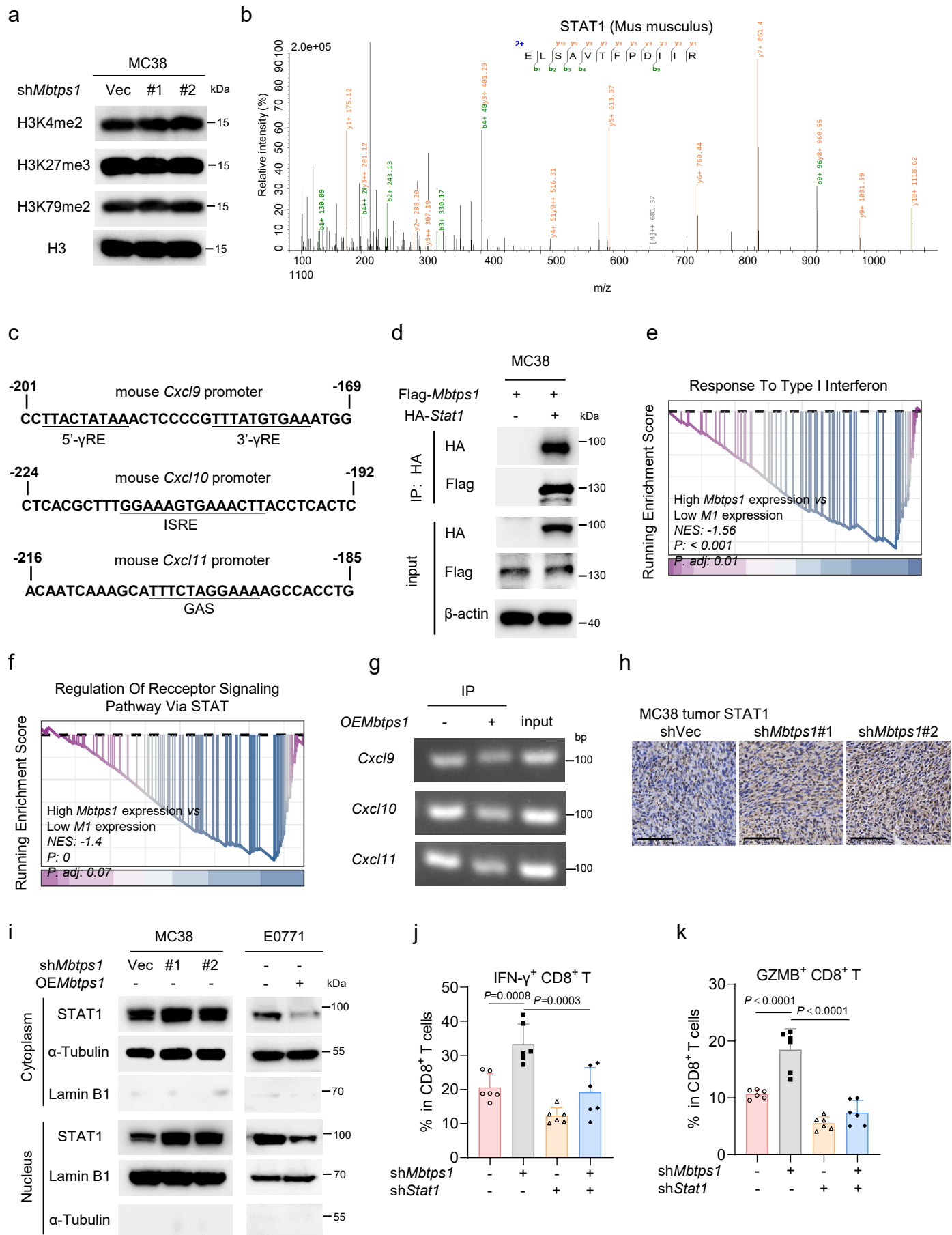
j qPCR analysis of *Cxcl9*, *Cxcl10* and *Cxcl11* mRNA levels in MC38 cells with *Mbtps1* overexpression.

k ELISA detection of CXCL9 and CXCL11 secreted into the culture medium by MC38 cells with *Mbtps1* knockdown.

l Percentages of Annexin-V positive MC38 cells with *Mbtps1* knockdown or overexpression after co-cultured with CFSE-labeled CD8⁺ T cells for 24 hours.

m Number of CXCR3⁺ cells in CD8⁺ T cells in CT26 tumors with *Mbtps1*-knockdown analyzed by flow cytometry.

n = 6 mice per group in **a** and **m**. n = 3 biologically independent samples per group, representative of three independent experiments with similar results in **j-l**. Data are presented as means ± SDs. Two-tailed unpaired Student's *t* test for **a**, **f** and **j-l**; normalized Enrichment Score (NES) and adjusted q-values (q<0.25) were computed utilizing the GSEA method, based on 1,000 random permutations of the ranked genes for **b**; DESeq2 with the FDR cutoff of 0.05 for **d**; Wilcox test for **e**.



Supplementary Fig. 6 MBTPS1 suppresses the production of chemokines by reducing the expression of STAT1.

a IB detection of H3K4me2, H3K27me3 and H3K79me2 expression in MC38 cells with *Mbtps1* knockdown.

b Representative spectrum of unique peptide of STAT1 in MBTPS1-immunoprecipitates. MC38 cells overexpressing Flag-MBTPS1 were immunoprecipitated with anti-FLAG tag antibodies, resolved on PAGE gel, and then subjected to LC/MS-MS analysis.

c The binding sites of STAT1 and the promoter regions of *Cxcl9/10/11*.

d Co-IP of HA-STAT1 with FLAG-MBTPS1 in MC38 cells.

e, f GSEA of the response to type I interferon pathway (**e**) and the regulation of receptor signaling pathway via STAT pathway (**f**), utilizing the TCGA-COAD dataset, demonstrating a comparison between high and low expression levels of *Mbtps1*.

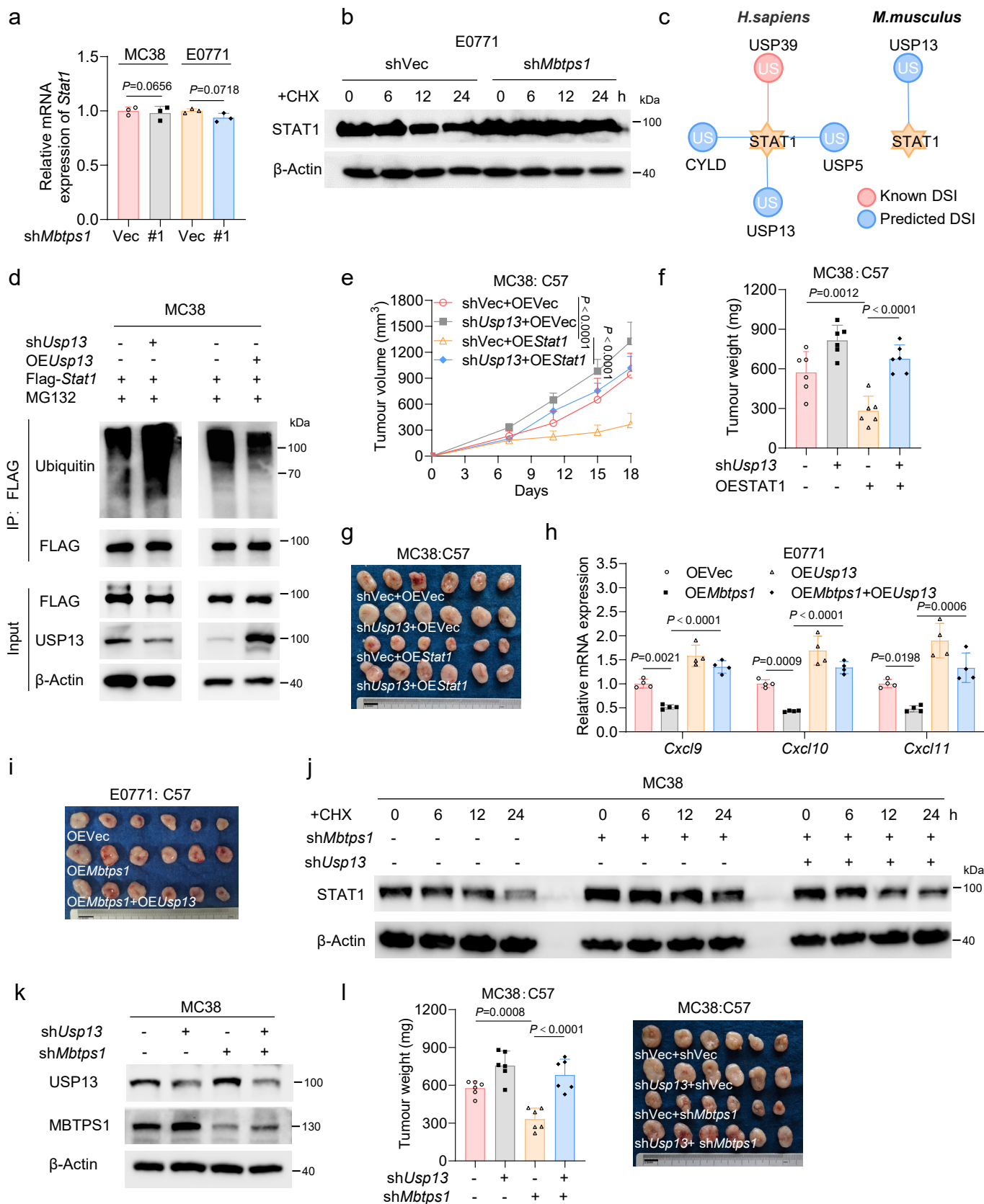
g ChIP-PCR analysis of the interaction between STAT1 and the *Cxcl9/Cxcl10/Cxcl11* promoter regions in MC38 cells after *Mbtps1* overexpression.

h Representative IHC staining images of STAT1 in MC38 tumors following *Mbtps1* knockdown. Scale bar: 100 μ m.

i The cytoplasmic and nuclear components were isolated from MC38 and E0771 cell lysates for IB detection of STAT1. α -Tubulin was included as a loading control for cytoplasmic extraction and Lamin B1 was included as a loading control for nuclear extraction.

j, k Flow cytometric analysis of the percentage of IFN- γ ⁺CD8⁺ T cells (**j**) and GZMB⁺CD8⁺ T cells (**k**) in E0771 tumors from indicated groups.

n = 6 mice per group in **j** and **k**. ChIP-PCR in **g** and IB experiments in **a**, **d**, and **i** were repeated three times with similar results using biologically independent samples. Data were presented as means $\pm$ SDs. One-way ANOVA with Sidak's multiple comparisons test for **j** and **k**; Normalized Enrichment Score (NES) and adjusted q-values (q<0.25) were computed utilizing the GSEA method, based on 1,000 random permutations of the ranked genes for **e** and **f**.



Supplementary Fig. 7 MBTPS1 disrupts the stability of STAT1 by competing with USP13 for binding to STAT1.

a qPCR analysis of *Stat1* mRNA expression in MC38 and E0771 cells with *Mbtps1* knockdown.

b IB detection of STAT1 expression in E0771 cells with *Mbtps1* knockdown followed by treatment with CHX (5 µg/ml).

c Schematic diagram of predicted the deubiquitinase-Substrate-interaction (DSI) of STAT1 using Ubrowser 2.0.

d Co-IP analysis of the interaction between FLAG-STAT1 and endogenous ubiquitin in MC38 cells with *Usp13* knockdown or overexpression.

e-g Growth curves (e), weight analysis (f) and tumor image (g) of MC38 tumors implanted in female C57 mice (6-8 weeks old) with knockdown of *Usp13* alone, overexpression of *Stat1* alone, and simultaneous knockdown of *Usp13* and overexpression of *Stat1*. Scale bar: 1 cm.

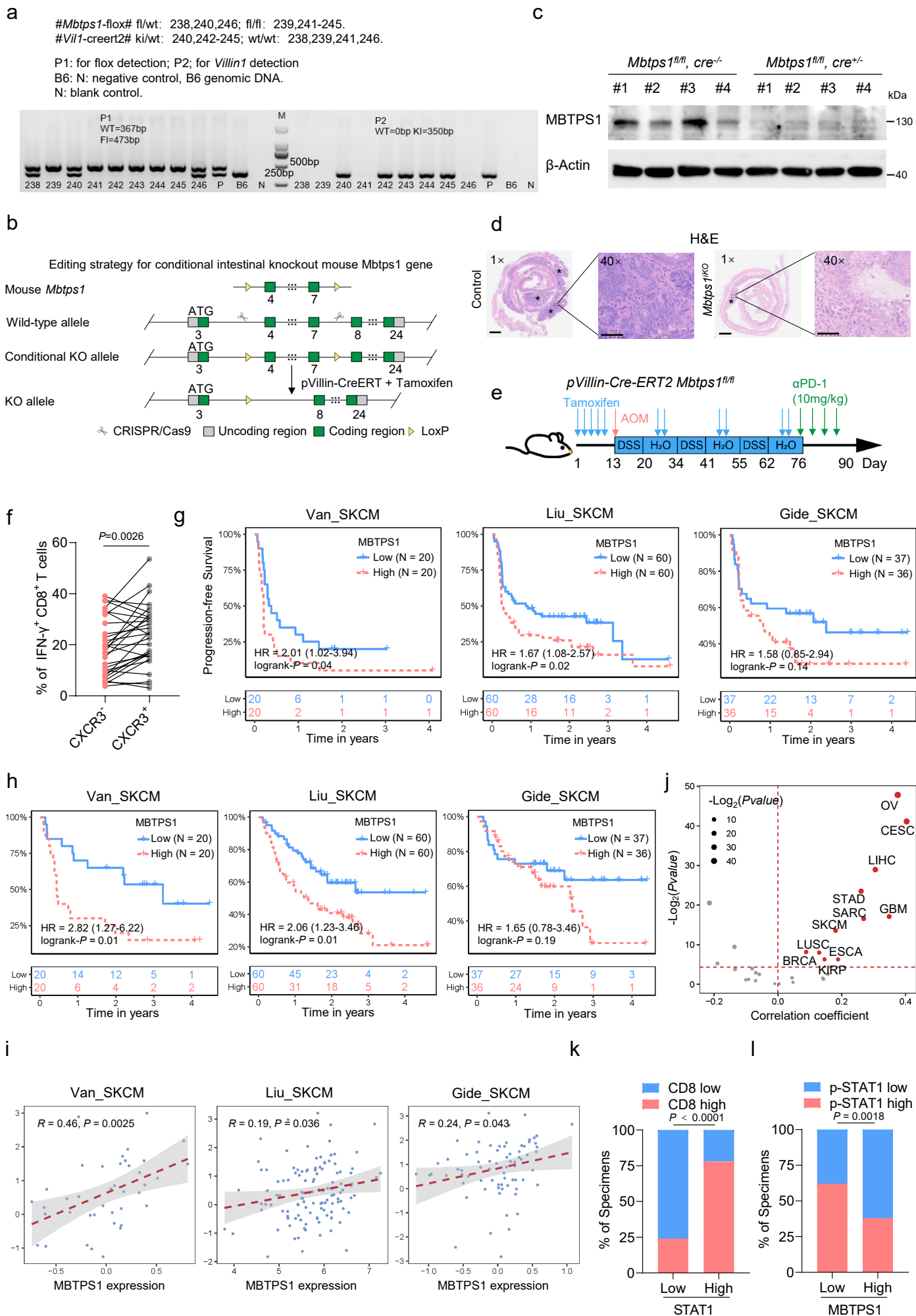
h qPCR analysis of *Cxcl9*, *Cxcl10* and *Cxcl11* mRNA levels in E0771 cells, in which *Mbtps1* was overexpressed alone, *Usp13* was overexpressed alone, or both *Mbtps1* and *Usp13* were overexpressed simultaneously.

i Tumor image of E0771 tumors with *Mbtps1* overexpression alone or both *Mbtps1* and *Usp13* overexpression. Scale bar: 1 cm.

j IB detection of USP13 and MBTPS1 of MC38 cells with indicated treatment.

k IB detection of STAT1 expression in MC38 cells after knockdown of *Mbtps1* alone, knockdown of *Usp13* alone, and simultaneous knockdown of both *Mbtps1* and *Usp13*, followed by treatment with CHX (5 µg/ml). βActin was included as a loading control.

l Weight analysis and tumor image of MC38 tumors with knockdown of *Mbtps1* alone, knockdown of *Usp13* alone, and simultaneous knockdown of both *Mbtps1* and *Usp13*. n = 3 biologically independent samples per group, representative of three independent experiments in **a** and **h**. n = 6 mice per group in **e-g**, **i** and **l**. IB experiments in **b**, **d**, **j** and **k** were repeated three times using biologically independent samples. Data were presented as means ± SDs. Two-tailed paired Student's *t* test for **a**; one-way ANOVA with Sidak's multiple comparisons test for **e**, **f**, **h** and **l**.



Supplementary Fig. 8 MBTPS1 drives tumor development and serves as a marker of unfavorable prognosis.

a Representative result image of PCR genotyping using mouse tail DNA. PCR screening for *Mbtps1* revealed a band of 367 bp for the wild-type allele and a band of 473 bp for the floxed allele.

b Editing strategy for conditional intestinal knockout of the mouse *Mbtps1* gene. Transgene expressing Cre recombinase under the control of the Villin gene promoter (villin-Cre-ERT2), an intestine-specific promoter.

c IB detection of MBTPS1 the in spontaneous tumors from female control and *Mbtps1*^{iKO} mice (6-10 weeks old). β -Actin was included as a loading control.

d Representative images of H&E staining in intestinal spontaneous tumor tissues of control and *Mbtps1*^{iKO} mice. Scale bar: 1 mm (4 $\times$); 50 μ m (40 $\times$). Asterisks mark the location of the intestinal tumor.

e Experimental design for the administration of anti-PD1 in AOM-DSS induced mouse CRC model.

f Percentages of IFN- γ positive cells in CXCR3⁺CD8⁺ T cells or in CXCR3-CD8⁺ T cells in AOM-DSS induced mouse CRC model.

g, h Correlation of MBTPS1 expression and the PFS (g) or OS (h) of patients treated with ICB (Melanoma_Van, n = 40; Melanoma_Liu, n = 120; Melanoma_Gide, n = 73).

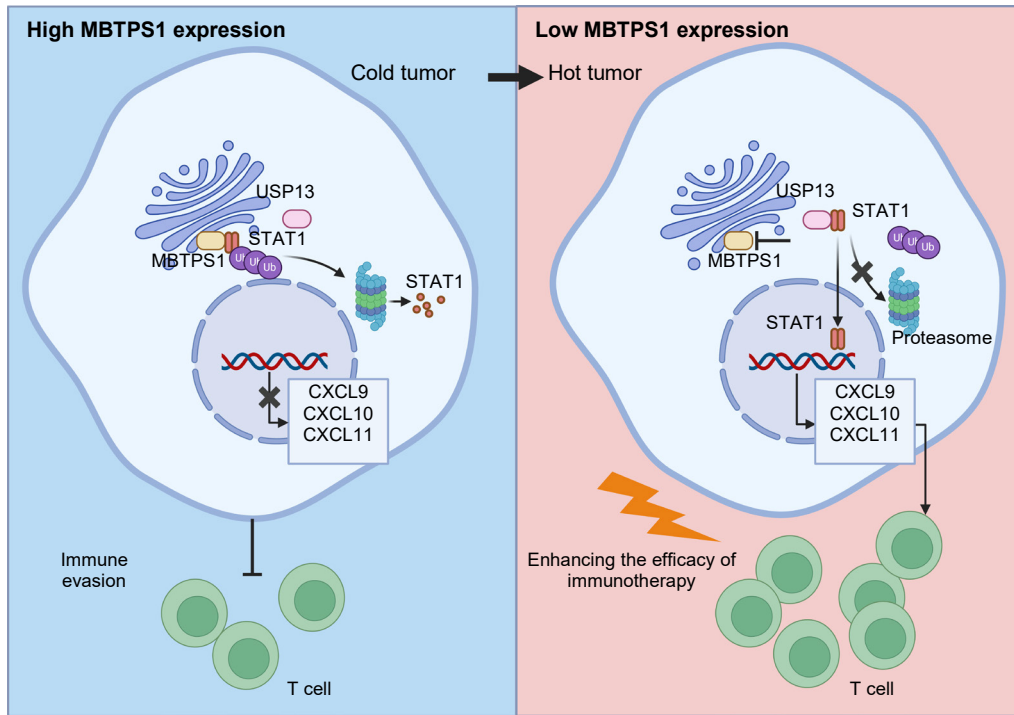
i Pearson correlation between TIDE prediction scores and MBTPS1 expression in the 3 cohorts mentioned in Supplementary Fig. 7g.

j Pearson correlation between TIDE prediction scores and MBTPS1 expression across 33 cancer types from TCGA.

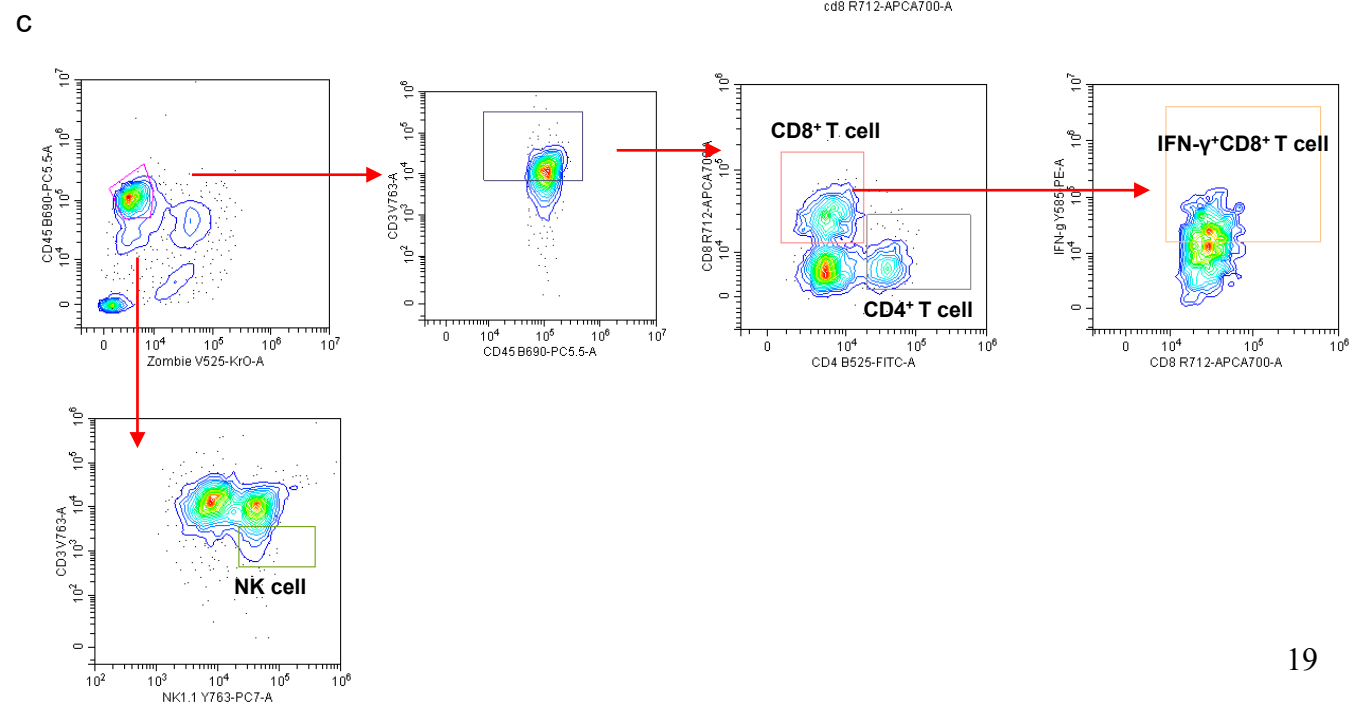
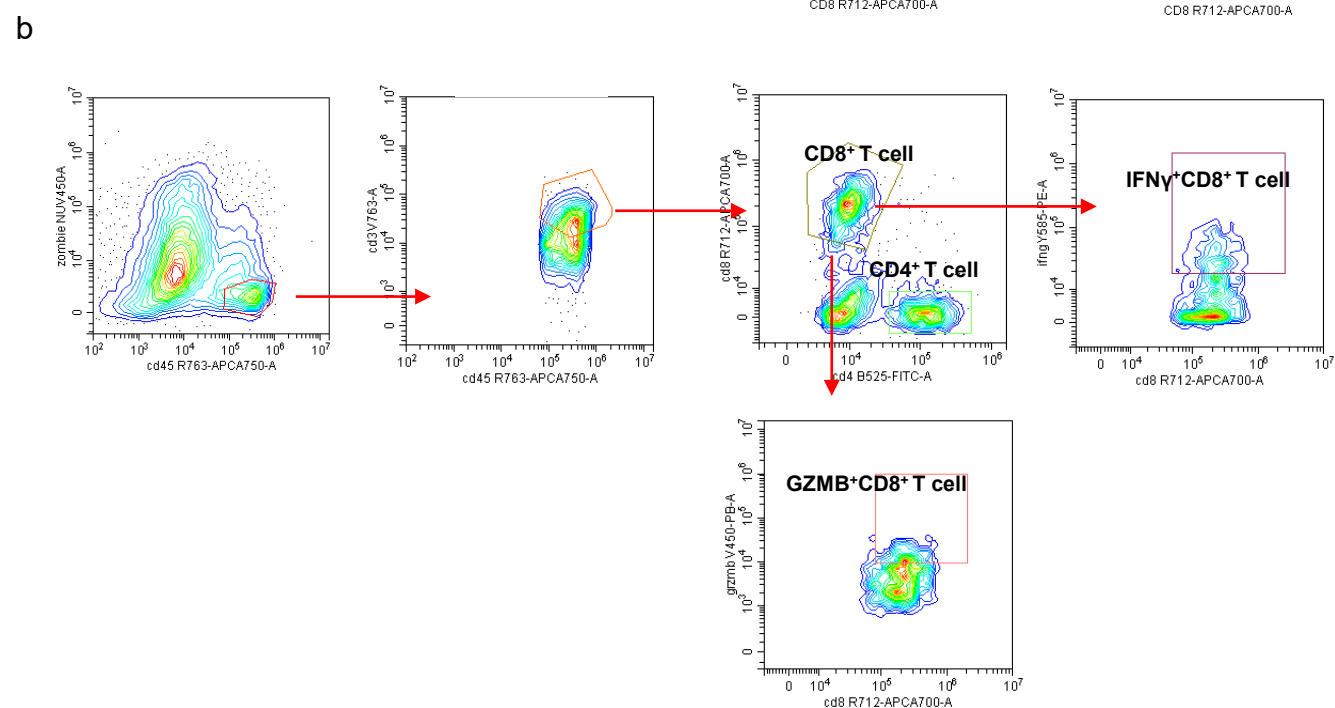
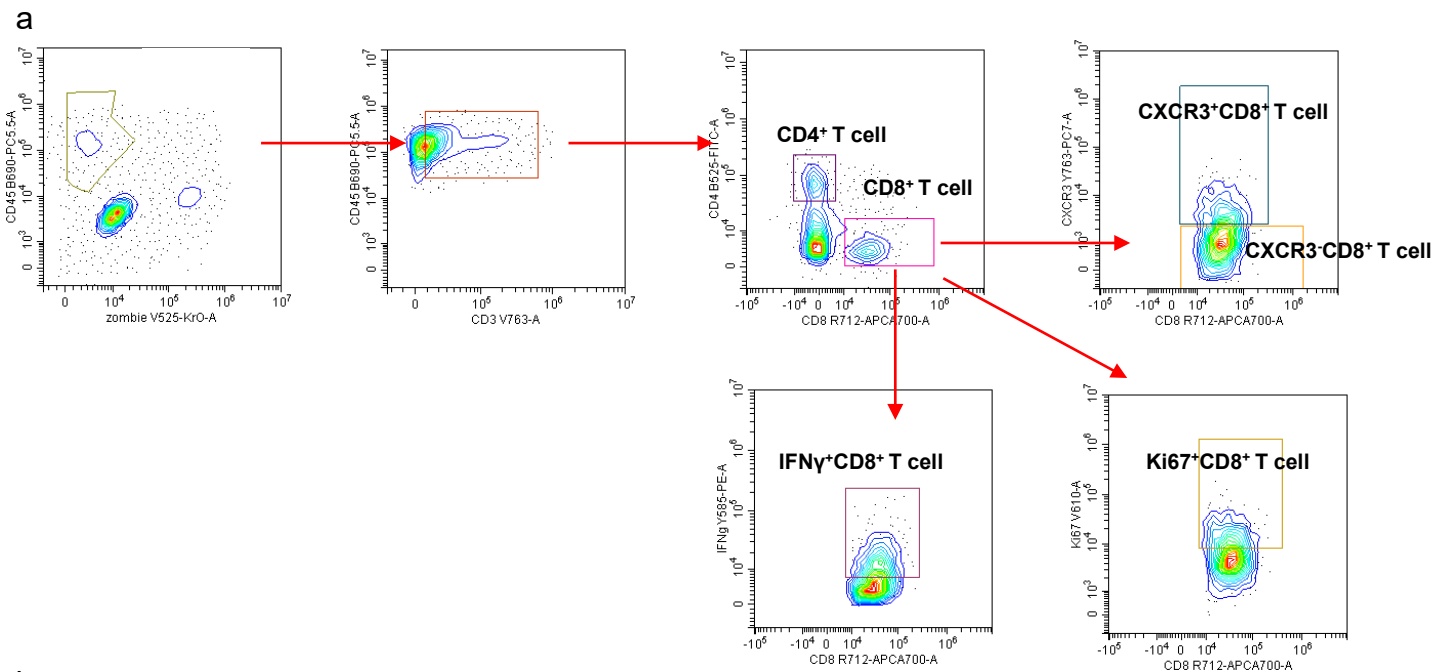
k Percentage of tumor tissues exhibiting high or low expression of CD8 presented for the group with low or high STAT1 expression.

l Percentage of tumor tissues exhibiting high or low expression of p-STAT1 presented for the group with low or high MBTPS1 expression.

Agarose gel electrophoresis for PCR genotyping in **a** and IB experiments in **c** are representative results of mice used in Figure 7a-l. n = 30 mice per group in **f**. Two-tailed paired *t* test for **f**; Two-sided pearson's correlation analysis and chi-square (and Fisher's exact) test for **k** and **l**; Log-rank test for **g** and **h**; Pearson correlation analysis for **i**; Spearman correlation analysis for **j**.



Supplementary Fig. 9 Working model of the proposed mechanism in this study. Arrows with gradient-faded tails indicate transport to functional sites. Solid-tailed arrows indicate generation relationships. Blunt-ended arrows indicate inhibitory effects. Bold arrow indicates transformation processes. Created in BioRender. Ji, H. (2025) <https://BioRender.com/w32d215>.



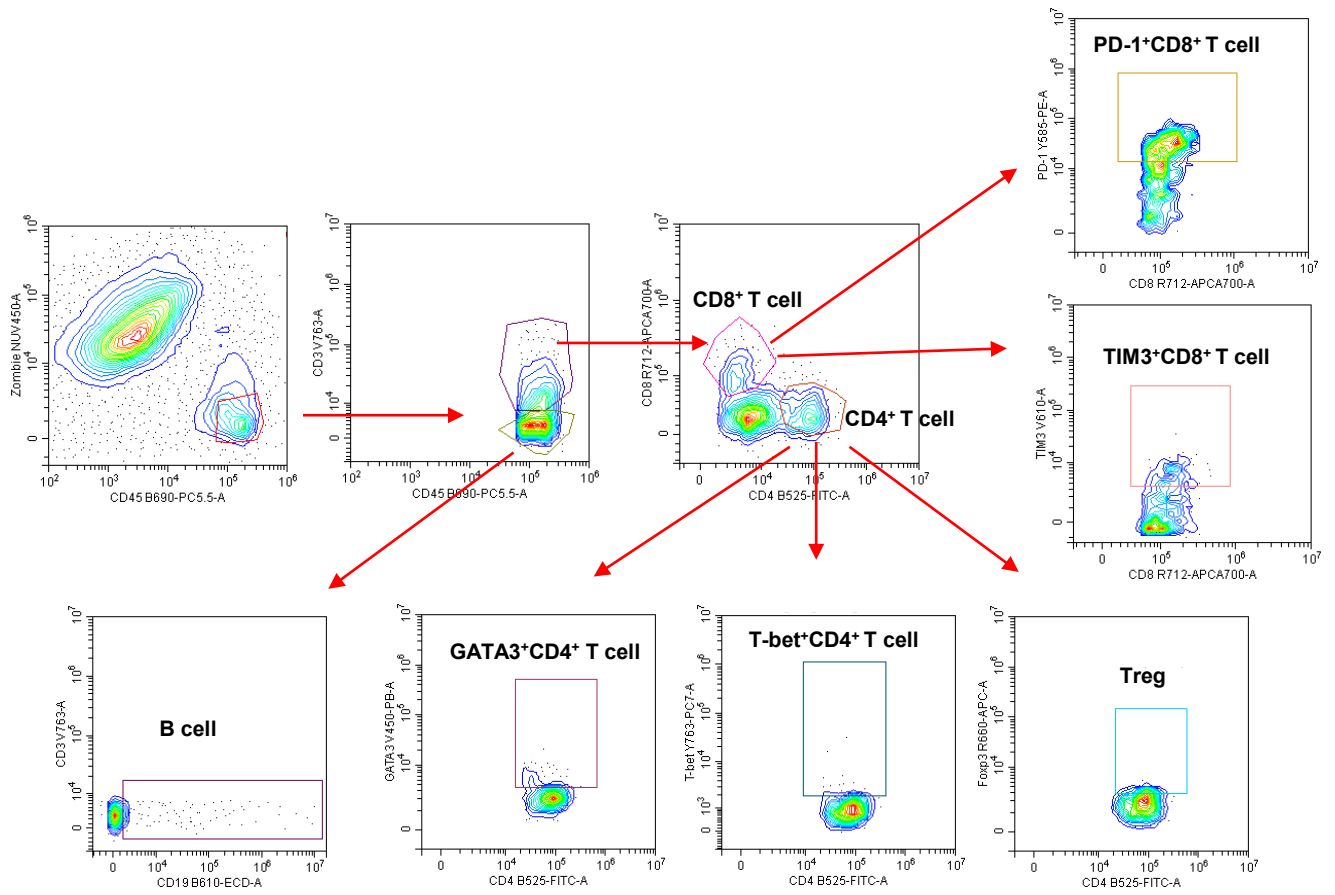
Supplementary Fig. 10 Gating strategy of flow cytometry assay for CD8⁺ T cell, CD4⁺ T cell, Ki67⁺CD8⁺ T cell, IFN γ ⁺CD8⁺ T cell, CXCR3⁺CD8⁺ T cell, CXCR3⁻CD8⁺ T cell and NK cell.

a Gating strategy for Figure 3e, 3g, 3h, 3i (right), 4m, 4p, 8g-k and Supplementary Figure 4m to sort CD8⁺ T cell, CD4⁺ T cell, Ki67⁺CD8⁺ T cell, IFN γ ⁺CD8⁺ T cell, CXCR3⁺CD8⁺ T cell and CXCR3⁻CD8⁺ T cell in CT26 tumors and in AOM/DSS-induced intestinal spontaneous tumor.

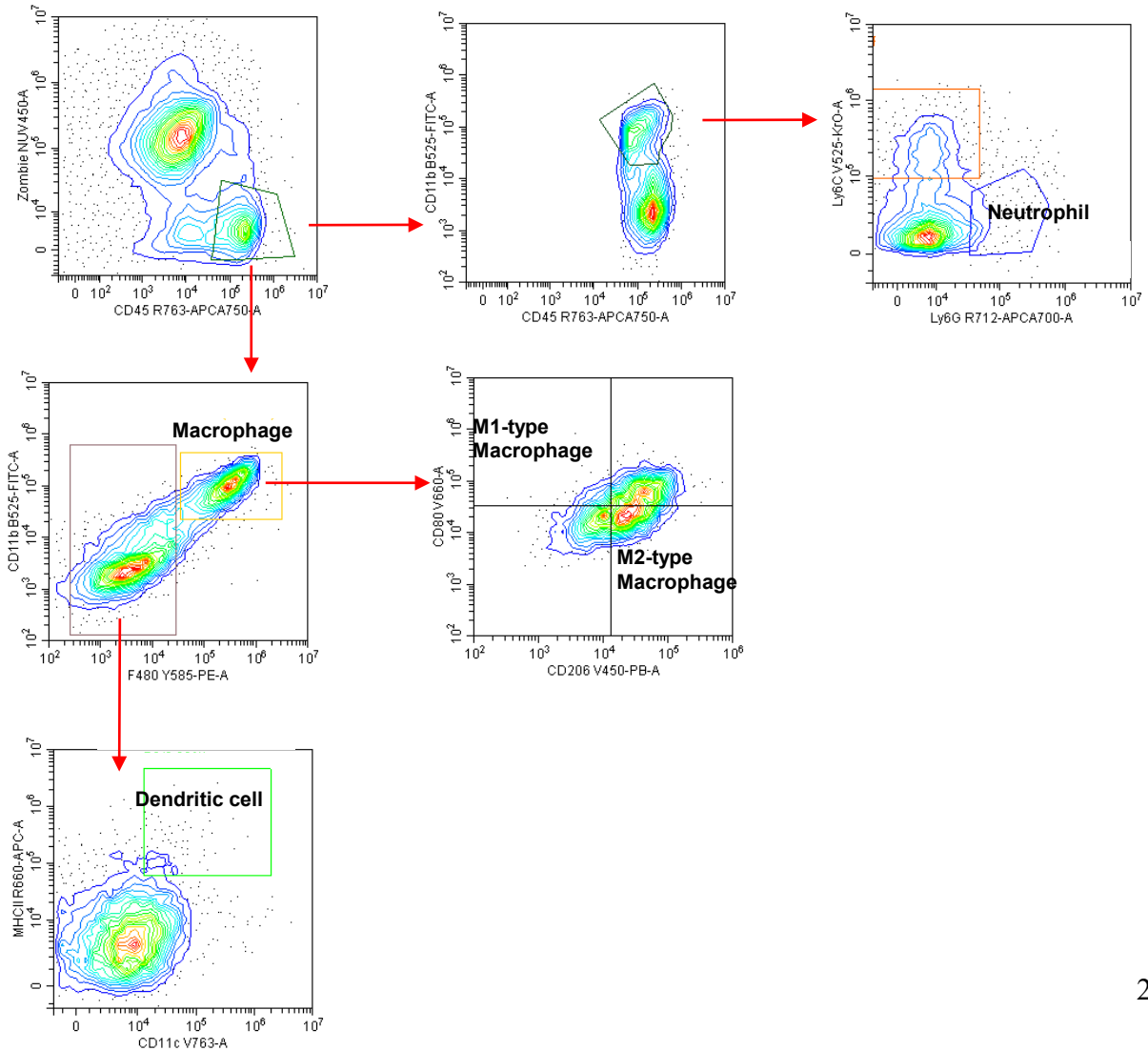
b Gating strategy for Figure 6l-n and Supplementary Figure 6j, 6k to sort CD8⁺ T cell, GZMB⁺CD8⁺ T cell and IFN γ ⁺CD8⁺ T cell in E0771 tumors.

c Gating strategy for Figure 3d, 3f, 3i, 3j and 3k to sort CD8⁺ T cell, CD4⁺ T cell, IFN γ ⁺CD8⁺ T cell and NK cell in MC38 tumors.

a



b



Supplementary Fig. 11 Gating strategy of flow cytometry assay for GATA3⁺CD4⁺ T cell, T-bet⁺CD4⁺ T cell, B cell, dendritic cell, macrophage and neutrophil.

a Gating strategy for Supplementary Figure 4f and 4g to sort GATA3⁺CD4⁺ T cell, T-bet⁺CD4⁺ T cell and B cell in MC38 tumors.

b Gating strategy for Supplementary Figure 4h-k to sort dendritic cell, macrophage and neutrophil in MC38 tumors.