Supplementary Figure Legends

Figure S1. RIG-I promotes immune evasion by suppressing T-cell antitumor immunity. A, Analysis of the efficiency of RIG-I mRNA knockdown or overexpression in HT29 cells, and RIG-I mRNA knockout or overexpression in CT26 and MC38 cells. B, Effects of RIG-I knockdown or knockout on cell proliferation in HT29 and CT26, as detected using CCK8 assays. C, D, Growth curves of CT26 cell-derived tumors from immunodeficient and immunocompetent mice. Tumors were measured at specified time points and then dissected at the endpoint (n = 8). E, Representative images and weights of tumors from WT and RIG-I-KO MC38 cells in nude mice (n = 8). F, Representative images and weights of tumors from WT and RIG-I-KO MC38 cells in C57BL/6 mice (n = 8). G, H, Growth curves of MC38 cell-derived tumors from immunodeficient and immunocompetent mice. Tumors were measured at specified time points and then dissected at the endpoint (n = 8). I, Representative pictures of H&E (hematoxylin-eosin)-stained lungs from the WT and RIG-I-KO groups. Scale bars, 50μm. J, Representative images and quantitative analysis of IHC of CD3+ and CD8+ T cells in CT26 cell-derived tumors taken from the indicated groups of BALB/c mice. K, L, Flow cytometry analysis of the frequency of CD3+ T-cell and CD8+ T cell subsets in PBMCs from lung metastasis in the model mice. M, N The indicated MC38-OVA cells were cocultured with T cells from OT-I mice for 48h (effector: target= 5:1) and tumor cell apoptosis was measured by flow cytometry. Data are shown as the mean ± SEM. All data are representative of three independent experiments (n=3). ns, no significant difference; * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Figure S2. RIG-I positively regulates PD-L1 protein expression and sensitizes colon cancer to immunotherapy. A, mRNA expression of type I IFN-associated genes and immune checkpoint genes in CT26 and MC38 cells upon stimulation with polyI:C (2μg/mL, 24h, n=3). B, mRNA expression of type I IFN-associated genes and immune checkpoint genes in CT26 and MC38 cells upon RIG-I overexpression. C, D, Protein expression of type I IFN-associated genes and immune checkpoint genes in CT26 and MC38 cells upon stimulation with polyI:C (2μg/mL, 24h) and RIG-I overexpression. E, IFN-α levels in the culture medium of HT29, CT26 and MC38 cells after stimulation with polyI:C (2μg/mL, 24h) and RIG-I overexpression, as determined by ELISA. F, PD-L1 mRNA levels in HT29 cells with RIG-I knockdown, CT26 and MC38 cells with RIG-I knockout. G, PD-L1, p-STAT1, and STAT1 protein levels in HT29 cells with RIG-I knockdown, CT26 and MC38 cells with RIG-I knockout. H, Representative protein staining and mean MFI of total PD-L1 in CT26 cells by flow cytometry (n=3).
Representative protein staining and MFI of cell surface PD-L1 on CT26 cells by flow cytometry (n=3).

H&E-stained sections of colon tissues from the orthotopic colon cancer model mice. Scale bars, 50μm.

RIG-I and PD-L1 protein expression as detected by western blot (K) and correlation analysis of western blot (L, left panel) and IHC score (L, right panel) in colon cancer tissue samples from Zhongnan Hospital (n = 14). All data are representative of three independent experiments (n=3). ns, no significant difference; * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Figure S3. RIG-I attenuates the polyubiquitination and proteasomal degradation of PD-L1 in colon cancer. A, PD-L1 protein levels in CT26 cells with RIG-I knockout that were treated with CHX (200ng/mL) for the indicated time (left panel). Graph showing the amount of PD-L1 protein that remained after CHX treatment (right panel). B, PD-L1 protein levels in CT26 cells with RIG-I knockout that were treated with MG132 (20μM) for 6h (left panel). Representative PD-L1 protein staining is shown in the right panel by flow cytometry (n=3). D, E, The polyubiquitination of PD-L1 was then examined by immunoprecipitation with a PD-L1 antibody in CT26 (D) and HEK293T cells (E) with RIG-I overexpression or knockout. Cells were treated with MG132 (20μM) for 6h before being harvested. F, The predicted conformation of a protein-protein complex between RIG-I (7TNX) and PD-L1 (3BIK) was predicted by docking using PyMol (v 2.2.0) and LigPlot+ (v 2.2.4).

Co-IP analysis of the interaction between endogenous and exogenous PD-L1 and RIG-I in CT26 (G) and HEK293T cells (H). I, Representative confocal microscopy images following IF analysis of RIG-I and PD-L1 expression and the colocalization of RIG-I (green) and PD-L1 (red) in CT26 cells. Scale bars, 10μm. All data are representative of three independent experiments (n=3). ns, no significant difference; * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Figure S4. RIG-I competes with SPOP to bind PD-L1, maintaining PD-L1 stability. A, B Representative confocal microscopy images following IF analysis of PD-L1 expression and the colocalization of PD-L1 (green) cell and cell membrane (dyed with Dil red probe) in HT29 and CT26 cells. Scale bars, 12.3μm. C, Flag-PD-L1 protein expression upon overexpression of Myc-SPOP and Myc-CUL1 in HEK293T cells. D, The polyubiquitination of FLAG-PD-L1 was then examined by co-IP with an anti-PD-L1 antibody in HEK293T cells transfected with Myc-SPOP. E, F, The expression of SPOP when RIG-I was overexpressed (E) or knocked down or knocked out (F) in HT29 and CT26 cells. G, Co-IP analysis of the interaction between exogenous FLAG-PD-L1, Myc-SPOP, and HA-RIG-I in
HEK293T cells. All data are representative of three independent experiments (n=3). ns, no significant difference; * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
Figure S1
Figure S2

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Figure S3

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Figure S3