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Supplemental Information

Deubiquitination and Stabilization of PD-L1 by CSN5

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SUPPLEMENTAL INFORMATION

SUPPLEMENTAL DATA

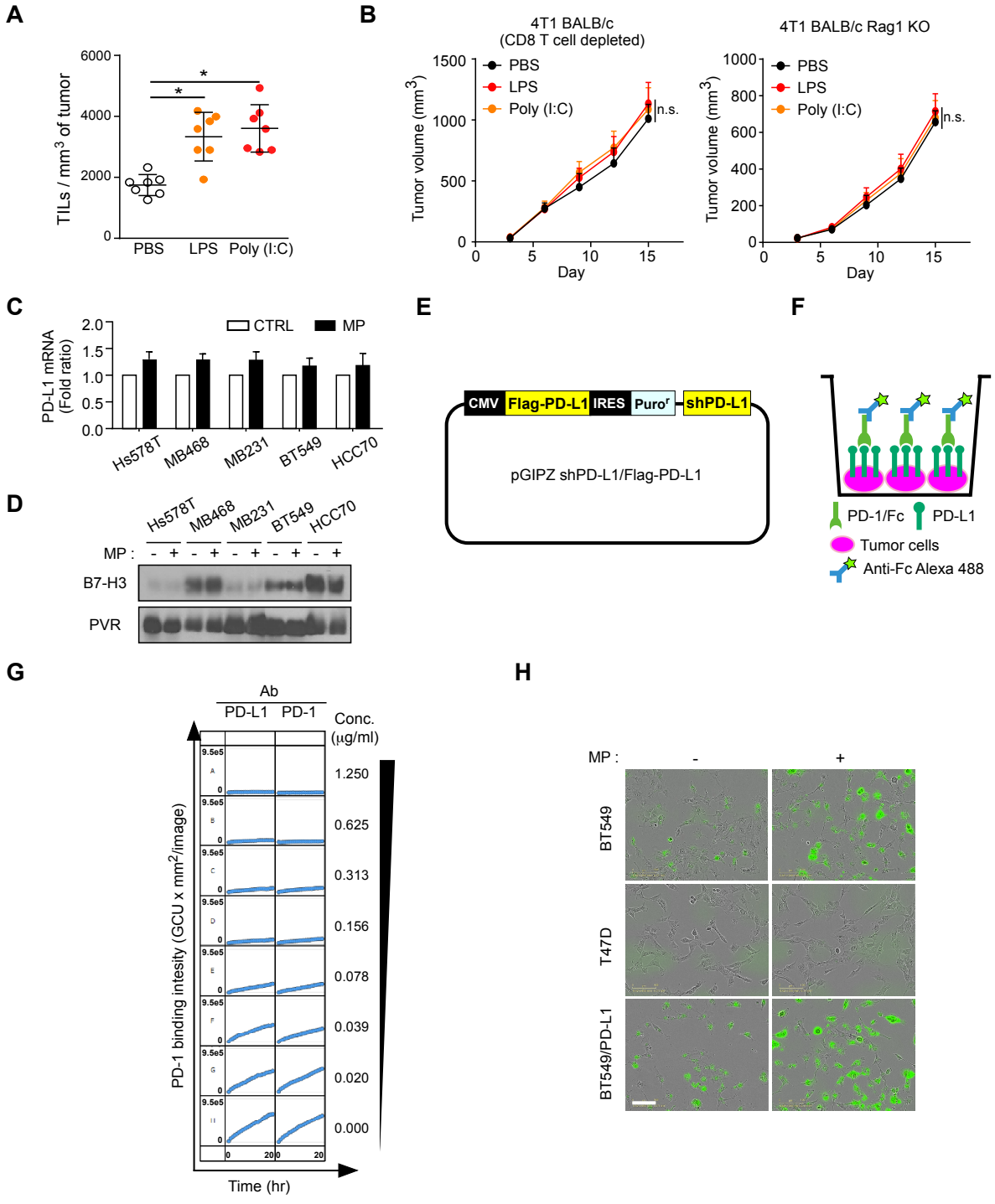

Figure S1, related to Figure 1. Macrophage-secreted inflammatory cytokines upregulate expression of PD-L1 protein. (**A**) Absolute number of tumor-infiltrating lymphocytes (TILs)

per cubic millimeter of tumor in PBS-, LPS-, or Poly (I:C)-treated BALB/c mice (n = 7 mice per group). Results are presented as mean ± SD from one representative experiment. *Statistically significant by Student's t test. (**B**) Tumor growth of 4T1 cells in $CD8^+$ T cell-depleted BALB/c mice (pretreated with CD8 antibody; left) or *Rag1* knockout (KO) BALB/c mice (right) treated with lipopolysaccharide (LPS), polycytidylic acid [Poly (I:C)], or phosphate-buffered saline (PBS) was measured at the indicated time points and dissected at the endpoint **(**n = 7 mice per group). Error bars are expressed as mean ± standard deviation. n.s., no significance. (**C**) qRT-PCR analysis of PD-L1 mRNA expression in breast cancer cells treated with macrophage-conditioned medium (MP). Error bars are expressed as mean ± SD of 3 independent experiments. (**D**) Western blot analysis of B7-H3 and PVR in breast cancer cells treated with MP. The loading control, tubulin expression was showed in Figure 1G. (**E**) Dual-expression construct for Flag-PD-L1 and shRNA of PD-L1. (**F**) Schematic diagram of the PD-L1/PD-1 interaction assay. Cells expressing PD-L1 were seeded on 96-well plates, and recombinant PD-1 Fc protein and anti-Fc Alexa 488 were then added and analyzed using IncuCyte ZOOM. (**G**) Kinetic graph showing the quantitative binding of PD-1/Fc protein on PD-L1-expressing BT549 cells at hourly time points after treatment with PD-L1 or PD-l neutralizing antibodies. (**H**) Representative images (at the 12-hour time point) of the binding described in Figure 1I. Scale bar, 100 μm.

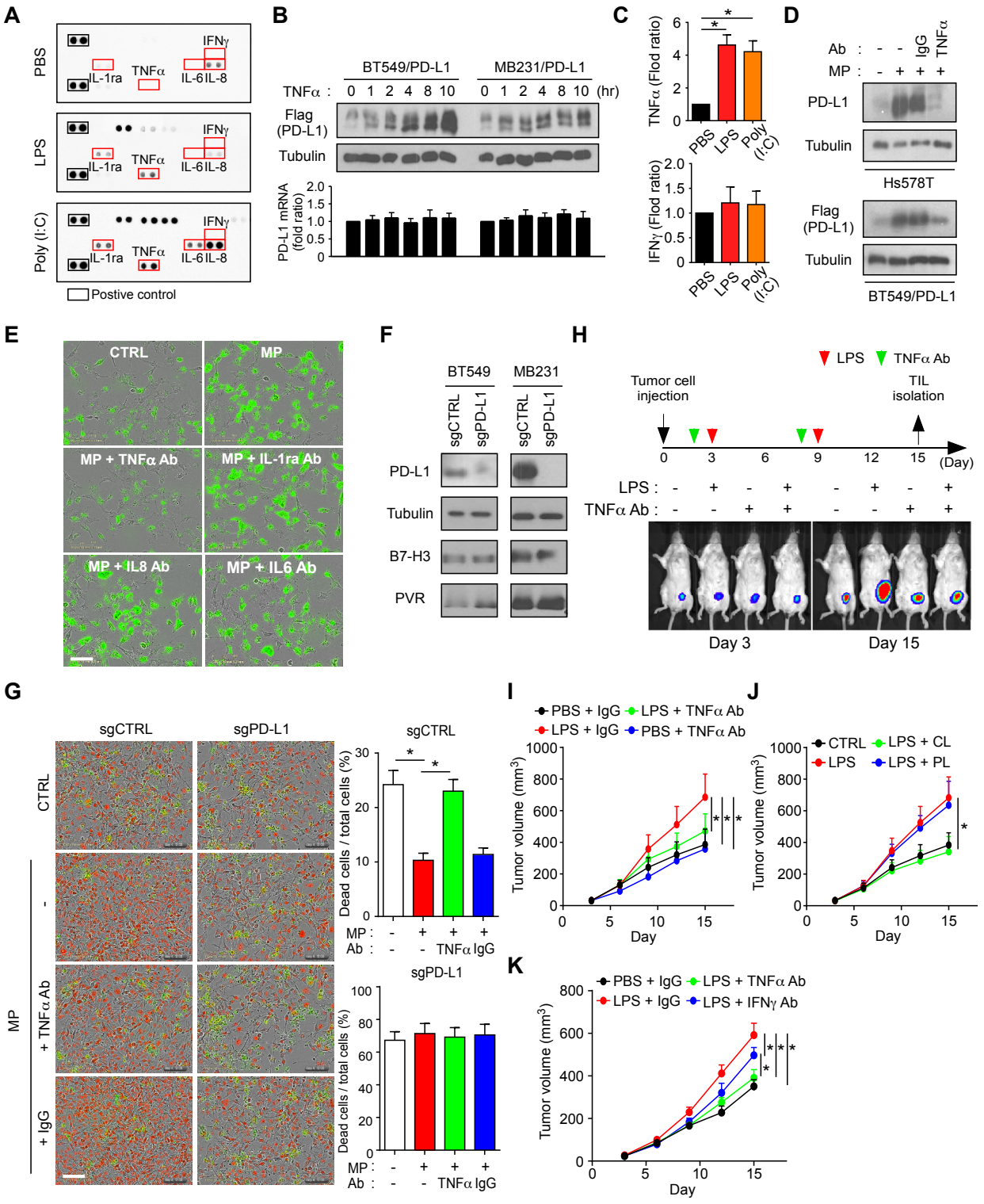

Figure S2, related to Figure 2. TNFα induces cancer immunosuppression via PD-L1 stabilization. (**A**) Analysis of secreted cytokines in phosphate-buffered saline (PBS)-,

lipopolysaccharide (LPS)-, or polycytidylic acid [Poly (I:C)]-treated macrophages by cytokine antibody array. The red box represents the 4 cytokines shown in Figure 2A. (**B**) PD-L1 expression analyzed using the Flag antibody in BT549/PD-L1 and MB231/PD-L1 cells that were serum-starved overnight before treatment with TNFα. PD-L1 mRNA expression (bottom) was analyzed using qRT-PCR. (**C**) The relative expression level of TNFα and IFNγ in PBS-, LPS-, or Poly (I:C)-treated 4T1 tumors at the 15-day time point. (**D**) Western blot analysis of PD-L1 in HS578T and BT549/PD-L1 cells treated with macrophage-conditioned medium (MP) and TNFα antibodies. (**E**) Representative images (at the 12-hour time point) of the binding experiment described in Figure 2C. Scale bar, 100 μm. (**F**) Western blot analysis of PD-L1 knockout in both BT549 cells and MDA-MB231 cells. (**G**) T cell-meditated tumor cell killing assay in PD-L1-knockout MDA-MB231 cells. Representative images (at the 4-day time point) of the MDA-MB231 cells are shown on the left and the quantitative ratio of dead cells is shown in the bar graph on the right. Scale bar, 100 μm. (**H**) Tumor growth of 4T1 cells in BALB/c mice following treatment with LPS and TNFα antibody. The treatment protocol is summarized by the arrows at the top. Tumor growth of 4T1 cells was visualized in vivo by bioluminescence imaging using IVIS100 (bottom). TIL, tumor-infiltrating lymphocyte. (**I**) Tumor growth of EMT6 cells in BALB/c mice was measured at the indicated time points and dissected at the endpoint (n = 7 mice per group). (**J**) Tumor growth of 4T1 cells in BALB/c mice with or without treatment with clodronate liposome (CL); n = 7 mice per group. (**K**) Tumor growth of 4T1 cells in BALB/c mice was measured at the indicated time points and dissected at the endpoint (n = 7 mice per group). *Statistically significant by Student's t test. All error bars are expressed as mean ± SD.

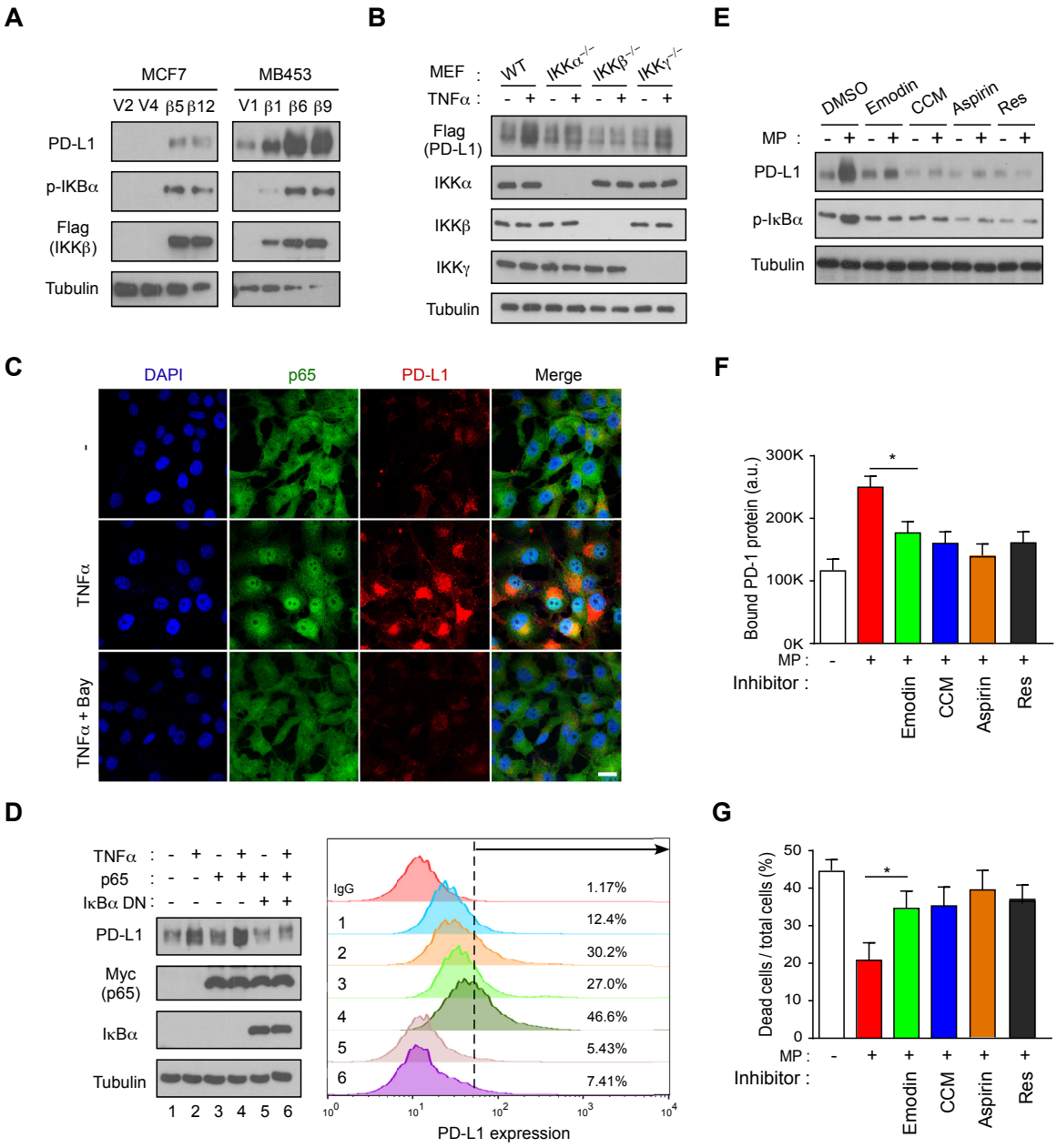

Figure S3, related to Figure 3. p65 activation regulates TNFα-mediated PD-L1 stabilization. (**A**) Western blot analysis of PD-L1 expression in IKKβ-expressing MCF7 and MDA-MB453 cells. V, vector control expressing clone; β, IKKβ-expressing clone. (**B**) PD-L1 expression in wild-type (WT), IKKα$^{-/-}$, IKKβ$^{-/-}$, or IKKγ$^{-/-}$ mouse embryonic fibroblasts (MEFs). Cells were transiently transfected with Flag-PD-L1 prior to treatment with TNFα and analyzed by Western blot analysis. (**C**) Confocal microscopy image showing p65 and PD-L1

expression in response to treatment with TNFα in MDA-MB231 cells. Cell nuclei staining by DAPI is shown on the left. Scale bar, 20 μm. (**D**) PD-L1 expression in p65- or dominant-negative (DN) IκBα-expressing cells. Cells were transiently transfected with the indicated plasmid prior to treatment with TNFα. Cell surface PD-L1 expression as analyzed by flow cytometry is shown on the right. IgG, isotype IgG control. (**E**) Western blot analysis of PD-L1 expression. BT549/PD-L1 cells were treated with emodin, curcumin (CCM), aspirin, or resveratrol (Res) overnight prior to treatment with macrophage-conditioned medium (MP). (**F**) PD-L1/PD-1 binding assay. BT549/PD-L1 cells treated with emodin, CCM, aspirin, or Res were examined using the PD-L1/PD-1 binding assay after treatment with MP. (**G**) T cell-meditated tumor cell killing assay in BT549 cells treated with emodin, CCM, aspirin, or Res. T cells were activated with CD3 antibody (100 ng/ml) and IL-2 (10 ng/ml). Green fluorescent cells were counted as dead cells. The quantitative ratio of dead cells is shown in the bar graph. *Statistically significant by Student's t test. All error bars are expressed as mean ± SD of 3 independent experiments.

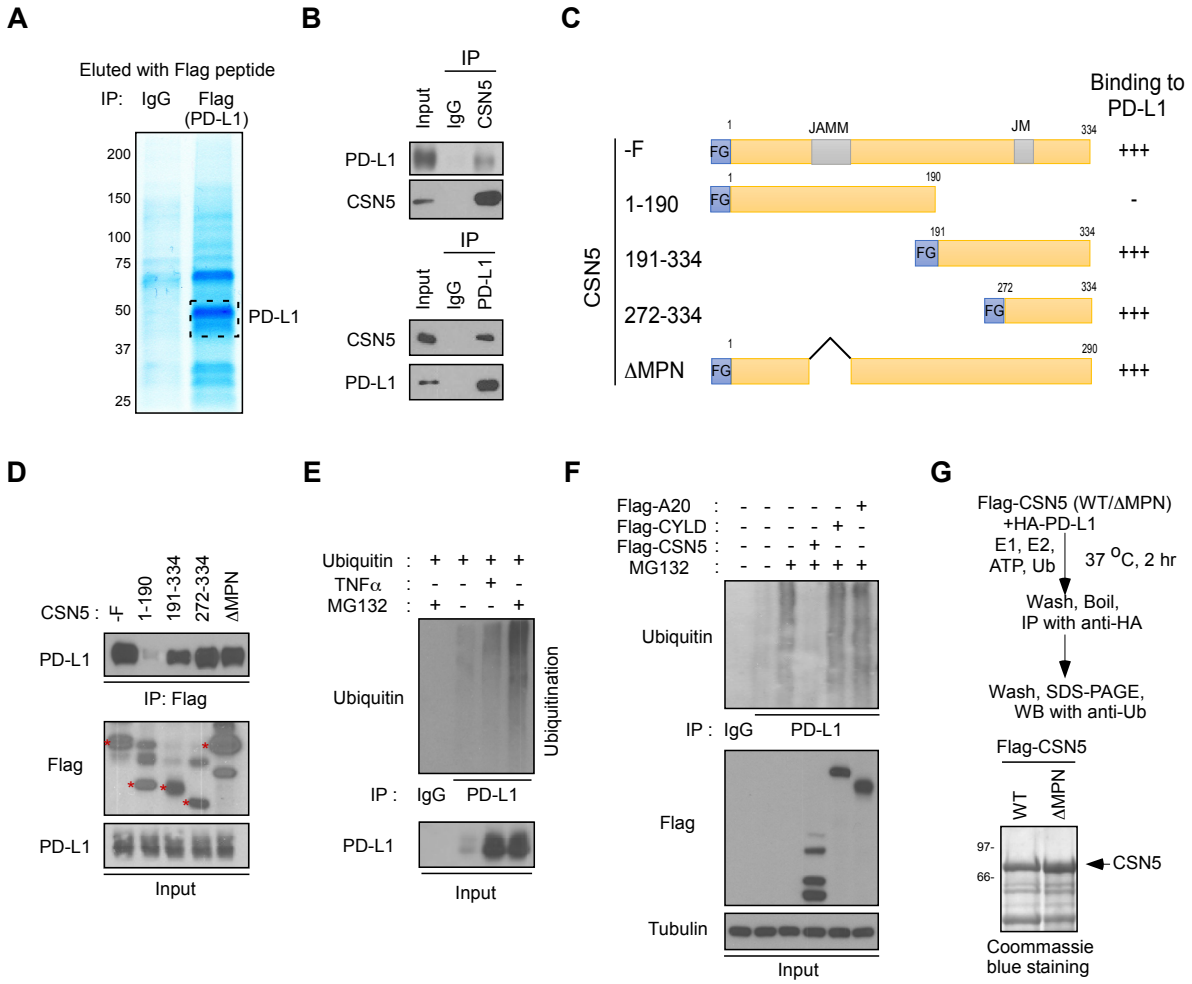

Figure S4, related to Figure 4. COP9 signalosome 5 (CSN5) induces PD-L1 stabilization. (**A**) Identification of PD-L1 binding proteins for LC-MS/MS analysis. Flag-PD-L1 protein was immunoprecipitated (IP) by Flag antibody-conjugated (M2) agarose resin and eluted by Flag peptide. The eluted Flag-PD-L1 binding protein was visualized using Coomassie brilliant blue. (**B**) Co-immunoprecipitation of PD-L1 and CSN5 in MDA-MB231 cells. Endogenous PD-L1 and CSN5 were immunoprecipitated and analyzed by Western blot analysis. IgG, negative control. (**C**) Schematic diagram of CSN5 deletion or truncation mutants used in the current study. The amino acid positions of individual fragments are labeled numerically. Binding to PD-L1 is shown on the right. (**D**) Co-immunoprecipitation of PD-L1 and CSN5 full-length (-F),

deletion (ΔMPN), or truncation mutants. (**E**) Ubiquitination of PD-L1 in BT549/PD-L1 cells. PD-L1 was IP with antibody and then subjected to Western blot analysis with the ubiquitin antibody. Cells were treated with MG132 or TNFα prior to ubiquitination analysis. (**F**) Western blot analysis of PD-L1 ubiquitination. BT549 cells were transiently transfected with HA-PD-L1 and Flag-A20, Flag-CYLD, or Flag-CSN5. PD-L1 proteins were IP and then subjected to Western blot analysis with the ubiquitin antibody. (**G**) Flow chart of in vitro deubiquitination assay.

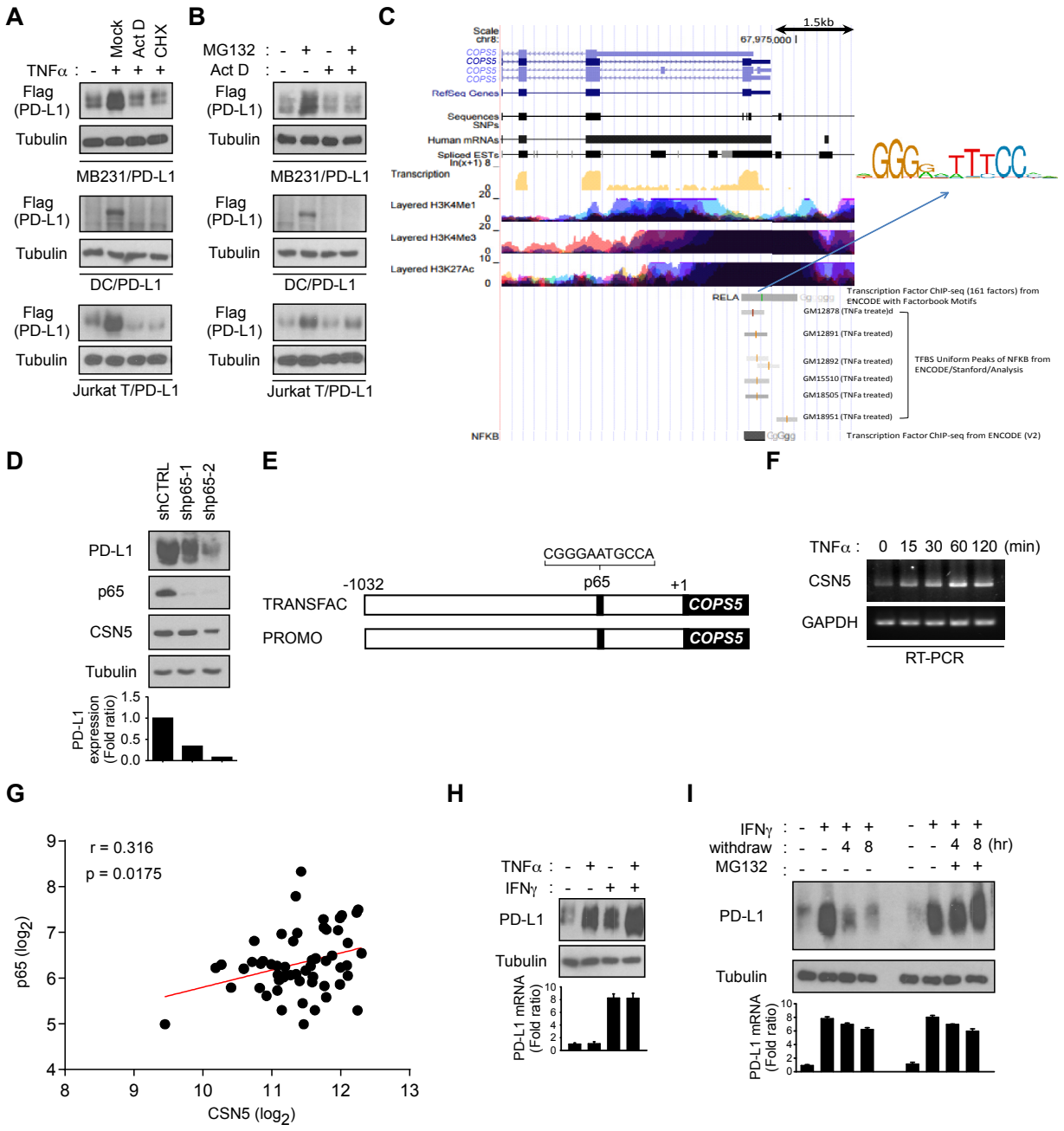

Figure S5, related to Figure 5. Transcriptional activation of CSN5 by p65 is required for TNFα-mediated PD-L1 stabilization. (**A**) PD-L1 expression detected by the Flag antibody in MB231/PD-L1, DC/PD-L1, or Jurkat T/PD-L1 cells pretreated with actinomycin D (Act D) or cycloheximide (CHX) for 5 hours. (**B**) PD-L1 expression detected by the Flag antibody in MB231/PD-L1, DC/PD-L1, or Jurkat T/PD-L1 cells pretreated with Act D or MG132 for 5 hours.

(**C**) ENCODE database analysis of p65 transcription factor on the *COPS5* core promoter region using chromatin immunoprecipitation sequencing after treatment with TNFα. (**D**) Western blot analysis of PD-L1, p65, and CSN5 proteins in 2 p65 knockdown clones. (**E**) p65 binding motif analysis of the *COPS5* promoter using the TRANSFAC and PROMO databases. (**F**) TNFα-mediated CSN5 transcriptional regulation. mRNAs isolated from TNFα-treated BT549 cells were subjected to RT-PCR using primer sets specific against CSN5 and GAPDH. (**G**) Correlation between p65 and CSN5 in the Cancer Cell Line Encyclopedia dataset. A positive correlation between p65 and CSN5 mRNA was found in breast cancer cell lines. (**H**) Western blot analysis of PD-L1 expression in TNFα and/or IFNγ treated BT549 cells. The expression of PD-L1 mRNA shows in bottom. (**I**) Western blot analysis of PD-L1 expression in BT549 cells treated with IFNγ and/or MG132. The expression of PD-L1 mRNA is shown (bottom). *Statistically significant by Student's t test. All error bars are expressed as mean ± SD of 3 independent experiments.

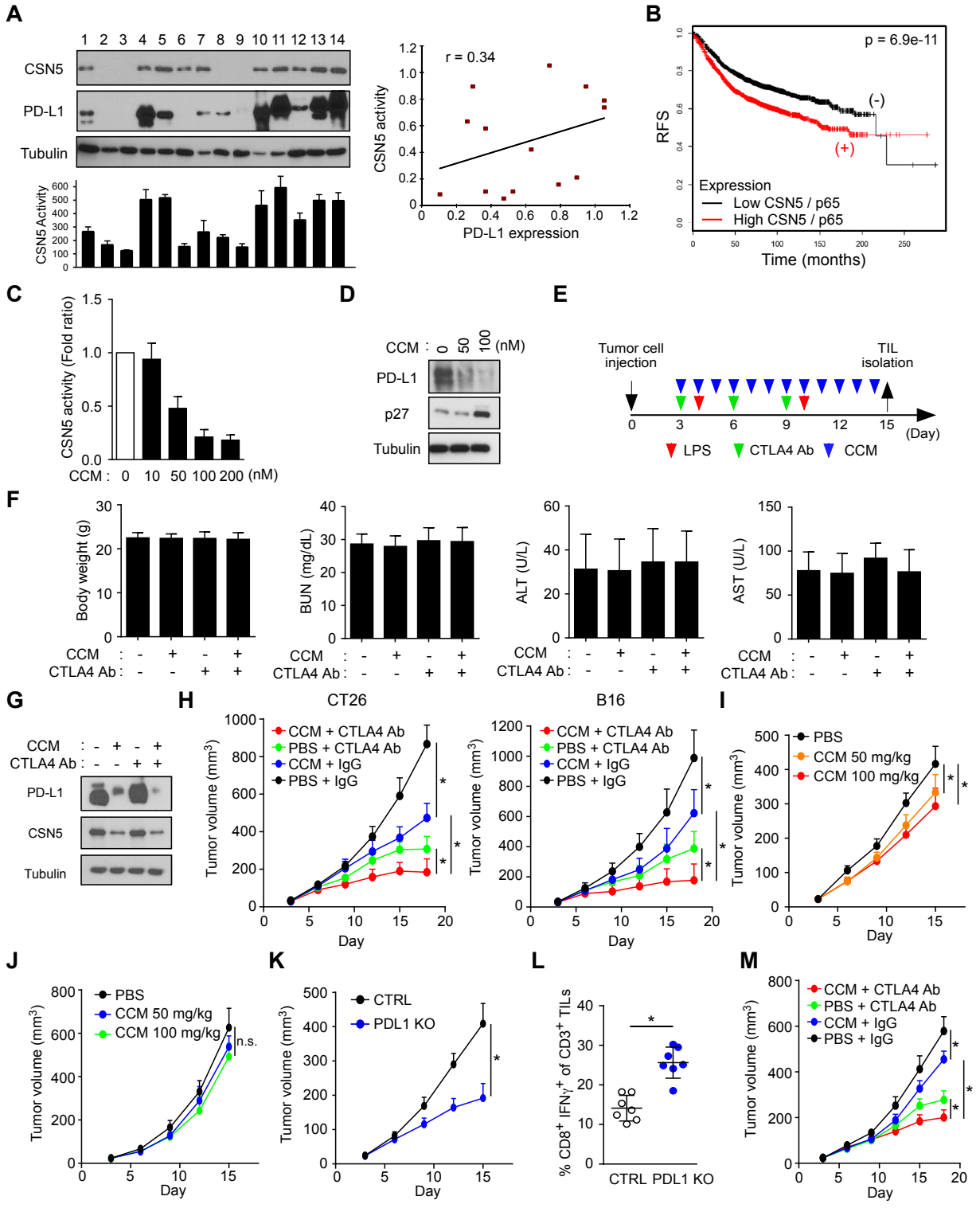

Figure S6, related to Figure 6. Inhibition of CSN5 enhances immunotherapy. (**A**) Western blot analysis of PD-L1 and CSN5 expression in breast cancer specimens (left) and correlation

between PD-L1 expression and CSN5 activity (right). (**B**) Recurrence-free survival (RFS) of patients with breast cancer with high and low p65 and CSN5 expression. A Kaplan-Meier plot was used for analysis. (**C**) CSN5 activity after treatment with curcumin (CCM) according to an in vitro deubiquitination assay. (**D**) Western blot analysis of PD-L1 and p27, a well-known target gene of CSN5, in CCM-treated MDA-MB231 cells. (**E**) Schematic diagram illustrating the treatment protocol of CCM and CTLA4 antibody in mice. LPS, lipopolysaccharide. (**F**) The effect of treatment on BALB/c mice. The body weight, mice liver, and kidney functions were measured at the end of the experiments. BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase. (**G**) Western blot analysis of PD-L1 and CSN5 expression in isolated 4T1 tumor cells from the experiments shown in Figure 6G. (**H**) Tumor growth of B16 and CT26 cells in C57BL/6J mice (B16 cells) or BALB/c mice (CT26 cells) treated with CCM or CTLA4 antibody following treatment with lipopolysaccharide. Tumors were measured at the indicated time points and dissected at the endpoint (n = 7 mice per group). (**I**) Tumor growth of mouse PD-L1 overexpressing 4T1 cells (4T1 mPD-L1) in BALB/c mice treated with 50 mg/kg or 100 mg/kg CCM (n = 7 mice per group). (**J**) Tumor growth of 4T1 cells in BALB/c Rag1 knockout (KO) mice treated with 50 mg/kg or 100 mg/kg CCM (n = 7 mice per group). n.s., no significance. (**K**) Tumor growth of PD-L1 knockout (KO) 4T1 cells in BALB/c mice (n = 7 mice per group). CTRL, parental 4T1 cells. (**L**) Intracellular cytokine staining of $CD8^+$ $IFN\gamma^+$ cells in $CD3^+$ T cell populations from isolated tumor-infiltrating lymphocytes. Results are presented as mean ± SD from one representative experiment. (**M**) Tumor growth of 4T1 cells in BALB/c mice treated with CCM or CTLA4 antibody (n = 7 mice per group). *Statistically significant by Student's t test. All error bars are expressed as mean ± SD unless otherwise noted.

Table S1, related to Figure 6. Correlations between expression levels of CSN5, PD-L1, phospho-p65 (p-p65), and granzyme B expression in surgical specimens of breast cancer analyzed using the PASS Pearson chi-square test.

	Number of Samples Expressing PD-L1 (%)			
Protein	**−/+**	**++/+++**	**Total**	**p value**
CSN5				0.0001
−/+	57 (67.1)	65 (39.9)	122 (49.2)	
++/+++	28 (32.9)	98 (60.1)	126 (50.8)	
Total	85 (100)	163 (100)	248 (100)	
p-p65				0.0001
−/+	53 (58.9)	59 (34.5)	112 (42.9)	
++/+++	37 (41.1)	112 (65.5)	149 (57.1)	
Total	90 (100)	171 (100)	261 (100)	
Granzyme B*				0.043*
−/+	50 (68.5)	92 (81.4)	142 (76.3)	
++/+++	23 (31.5)	21 (18.6)	44 (23.7)	
Total	73 (100)	113 (100)	186 (100)	

*An inverse correlation between p-p65 and Granzyme B.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell culture, stable transfectants, and transfection

All cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and were independently validated by short tandem repeat DNA fingerprinting at The University of Texas MD Anderson Cancer Center (Houston, TX, USA). These cells were grown in DMEM/F12 or RPMI 1640 medium supplemented with 10% fetal bovine serum. PD-L1 stable transfectants in MDA-MB-231 (MB231), MDA-MB-468 (MB468), BT549, and HEK293T cells were selected using puromycin (InvivoGen, San Diego, CA, USA). IKK$\alpha^{-/-}$, IKK$\beta^{-/-}$, and p65$^{-/-}$ mouse embryonic fibroblasts were maintained as previously described (Li et al., 2012). For transient transfection, cells were transiently transfected with DNA using X-tremeGENE (Roche Diagnostics, Indianapolis, IN, USA) or lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). For TNFα or IFNγ treatment, cells were serum starved overnight prior to cytokines stimulation at the indicated time points.

Animal treatment protocol

All procedures with BALB/c mice (6- to 8-week-old females; Jackson Laboratories, Bar Harbor, Maine, USA) were conducted under guidelines approved by the Institutional Animal Care and Use Committee at MD Anderson. Mice were divided according to the mean tumor volume in each group. 4T1 or EMT6 cells (5×10^4 cells in 50 μL of medium mixed with 50 μL of Matrigel Basement Membrane Matrix [BD Biosciences, San Jose, CA, USA]) were injected into the mammary fat fad. On days 4 and 10 after the inoculation, mice were injected intraperitoneally with 10 μg of LPS (Sigma-Aldrich, St. Louis, MO, USA) in PBS or with PBS alone. For treatment with antibodies, 100 μg of TNFα antibody (XT3.11; Bio X Cell, West Lebanon, NH,

USA), CTLA-4 antibody (Bio X cell), or control rat IgG (Bio X Cell) was injected intraperitoneally on days 3, 6, and 9 after tumor cell inoculation. For drug treatment, mice were treated with daily oral dose 50 mg/kg curcumin (Sigma-Aldrich) for two weeks from day 3 after tumor cell inoculation. For macrophage depletion (Elsegood et al., 2015; Van Rooijen and Sanders, 1994), 100 μL of clodronate liposome (ClodronateLiposomes.com, The Netherlands) or control PBS liposome (ClodronateLiposomes.com) was injected intraperitoneally on days 3 and 6 after tumor cell inoculation. Tumors were measured every 3 days with a caliper, and tumor volume was calculated using the following formula: $\pi/6 \times \text{length} \times \text{width}^2$.

Generation of stable cells using lentiviral infection

The lentiviral-based shRNA (pGIPZ plasmids) used to knock down expression of human or mouse PD-L1 (Shen et al., 2013) was purchased from the shRNA/ORF Core Facility at The University of Texas MD Anderson Cancer Center (Houston, TX, USA). On the basis of knockdown efficiency of PD-L1 protein expression in MDA-MB-231 or A431 cells, we selected 2 shPD-L1 clones for the current study. The mature antisense sequences were TCAATTGTCATATTGCTAC (shPD-L1 #1) and TTGACTCCATCTTTCTTCA (shPD-L1 #5). Using a pGIPZ-shPD-L1/Flag-PD-L1 dual-expression construct to knock down endogenous PD-L1 and reconstitute Flag-PD-L1 simultaneously (Lim et al., 2016), we established endogenous PD-L1 knockdown and Flag-PD-L1 wild-type (WT)-expressing cell lines. To generate lentivirus-expressing shRNA for PD-L1 and Flag-PD-L1, we transfected HEK293T cells with pGIPZ-non-silence (for vector control virus), pGIPZ-shPD-L1, or pGIPZ-shPD-L1/ PD-L1 WT with X-tremeGENE transfection reagent (Roche Diagnostics, Mannheim, Germany). Twenty-four hours after transfection, the medium was changed and then collected at 24-hour intervals.

The collected medium containing lentivirus was centrifuged to eliminate cell debris and filtered through 0.45-μm filters. Cells were seeded at 50% confluence 12 hours before infection, and the medium was replaced with medium containing lentivirus. After infection for 24 hours, the medium was replaced with fresh medium and the infected cells were selected with 1 μg/ml puromycin (InvivoGen, San Diego, CA, USA). For PD-L1 knockout, we transfected human or mouse PD-L1 double nickase plasmid (Santa Cruz Biotechnology, Dallas, TX, USA) into 4T1, EMT6, BT549, or MDA-MB231 cells using X-tremeGENE transfection reagent. For mouse PD-L1 overexpression 4T1 cells (4T1-mPD-L1), we infected mouse PD-L1 KO 4T1 cells with lentiviurs carrying pGIPZ-shmPD-L1/mPD-L1 followed by selection with puromycin. For *CSN5* knockout, we transfected cells with the mouse CSN5 double nickase plasmid (Santa Cruz Biotechnology) using X-tremeGENE transfection reagent. After transfection for 24 hours, the medium was replaced with fresh medium, and the transfected cells were selected with puromycin (InvivoGen). For endogenous CSN5 overexpression, 4T1 cells were infected with CSN5 CRISPR activation lentivirus (Santa Cruz Biotechnology). After infection for 48 hours, the medium was replaced with fresh medium, and the transfected cells were selected with puromycin (InvivoGen).

Antibodies and chemicals

The antibodies used in the current study are listed below. TNFα, IL-6, IL-8, and IL-1ra were purchased from R&D Systems (Minneapolis, MN, USA). Cycloheximide, lipopolysaccharide, curcumin, emodin, aspirin, and resveratrol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polycytidylic acid was purchased from InvivoGen. SB2035580, PD89059, LY294002, U0126, and Bay 11-7082 were purchased from Cell Signaling Technology (Denvers, MA, USA).

Antibodies used in Western blot analysis, immunofluorescence, and immunoprecipitation:

Antibody	Company	Catalog number
Flag	Sigma-Aldrich	F3165
Flag	Cell Signaling Technology*	2368
Myc	Roche Diagnostics	11667203001
HA	Roche Diagnostics	11666606001
PD-L1	Cell Signaling Technology	13684
PD-L1	BioLegend	329702
PD-L1	R&D Systems	AF157
PD-1	Abcam†	ab52587
PD-1	BioLegend	329911
Lamin B	Abcam	ab16048
Tubulin	Sigma-Aldrich	B-5-1-2
β-Actin	Sigma-Aldrich	A2228
TNFα	R&D Systems	AF210
Granzyme B	Abcam	ab4059
p65	Santa Cruz Biotechnology	SC-372
p-p65	Abcam	ab86299
CSN2	Abcam	ab10426
CSN5	Santa Cruz Biotechnology	SC-13157
CSN5	Cell Signaling Technology	6895
CSN5	Bethyl Laboratories‡	IHC-00362
AKT	Cell Signaling Technology	9272
p-AKT	Cell Signaling Technology	4060
p27	Cell Signaling Technology	3686
p-IκBα	Cell Signaling Technology	2859
IKKα	Cell Signaling Technology	11930
IKKβ	Cell Signaling Technology	8943
IKKγ	Cell Signaling Technology	2695
EGFR	Cell Signaling Technology	4267
B7H3	Cell Signaling Technology	14058
PVR	Cell Signaling Technology	13544

*Denvers, MA, USA.

†Cambridge, MA, USA.

‡Montgomery, TX, USA.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed as described previously(Li et al., 2012). Briefly, cells were incubated with 1% formaldehyde in cell culture media for 20 minutes at 37°C. Thereafter, the cross-linked cells were washed twice with ice-cold phosphate-buffered saline and lysed in SDS

buffer containing 1% SDS, 10mM EDTA, 50mM Tris-HCl (pH 8.1), and protease inhibitor cocktail (Roche Diagnostics). Nuclear extracts were sonicated. Supernatants were then diluted in 10-fold dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl [pH 8.1], and 16.7mM NaCl) and pre-cleared with 80 μl salmon sperm DNA-protein A-agarose (EMD Millipore, Billerica, MA, USA) for 2 hours at 4 °C. The sheared DNA mixture was subjected to immunoprecipitation with 1 μg p65 antibody (F3165; Sigma-Aldrich), RNA polymerase II antibody (05-623B; EMD Millipore), or an equivalent amount of normal mouse immunoglobulin G (Santa Cruz Biotechnology) overnight at 4°C. The protein-DNA complexes were then precipitated, washed, and eluted for PCR analysis.

Quantitative reverse transcription (qRT) PCR assays

qRT-PCR assays were performed to measure the expression of mRNA (Chang et al., 2011; Shen et al., 2013). Cells were washed twice with phosphate-buffered saline and immediately lysed in QIAzol. The lysed sample was subjected to total RNA extraction using the RNeasy Mini Kit (Qiagen, Hilden, Germany). To measure the expression of mRNA, we synthesized cDNA from 1~2 μg purified total RNA using the SuperScript VILO cDNA synthesis kit (ThermoFisher Scientific, Pittsburg, PA, USA) according to the manufacturer's instructions. qPCR was performed in a real-time PCR machine (iQ5, BioRad, Hercules, CA, USA) using the primers listed below). All data analysis was performed using the comparative Ct method. Results were first normalized to internal control β-actin mRNA.

Primers sequences (5′ to 3′):

β-actin	Forward	GCAAAGACCTGTACGCCAACA
	Reverse	TGCATCCTGTCGGCAATG
CSN1	Forward	AAGATGCTGGACGAGATGAAG
	Reverse	GGGCTGAAATACTGGATGAGG

CSN2	Forward	AGTAACTCCGAGCCAAATGTG
	Reverse	GTGCTTTAAATCCCCATTCTCC
CSN3	Forward	CTTATCCCATCTGGACACTGTG
	Reverse	ATGAAGAGCTGAACCTGTGAG
CSN4	Forward	AACCGAAGGACGTATGAATGG
	Reverse	GGTGCTGTTTGACTAATTTTCTCC
CSN5	Forward	CACTGAAACCCGAGTAAATGC
	Reverse	ACATCAATCCCAGAAAGCCAG
CSN6	Forward	CTGGAGTTTCTGGGTTGGTAT
	Reverse	TGTGCTTGGTCATAGGGTTC
CSN7A	Forward	AATAAGCTTCGACACCTCTCAG
	Reverse	CATACACAGCCTCAATCACAAG
CSN7B	Forward	GGACATACCCAGATTACATAGCC
	Reverse	TCTTTCAGCAACACGGAGTAG
CSN8	Forward	AGACGGTCCAGCCAATTATG
	Reverse	TGCCTTTCACAGCCTCTTC
USP1	Forward	GCGTTGCTTGGAATGTGAAAG
	Reverse	CCATCTCAGGGTCTTCATTTCTG
USP2	Forward	CCTAAGTCCAACCCTGAGAAC
	Reverse	ACCACAATCTGTACACGTCAG
USP3	Forward	CGTGGAGTTAAGGAATGGGAA
	Reverse	AAGGACTCTGGGCTAAATGC
USP4	Forward	CAGGGTCAGGTGCTAGTAATTG
	Reverse	CAGAGCCCAGGTTGTATATGAG
USP5	Forward	GGAAGTATGTGGATAAGCTGGAG
	Reverse	CGGTACTGGCTTGGAATACTC
USP6	Forward	ACCTTCGCTATCAAACATCCC
	Reverse	GTACATCTCACTTTCCCCTAGC
USP7	Forward	CCAGGAGAAGGAGTTTGAGAAG
	Reverse	GGCCGAGGATGAGACATATTAC
USP8	Forward	AAGGCAGGAAACAGGAAGAG
	Reverse	CTGTGATTGCTCCTTTCTCTTTG
USP10	Forward	GATCAATAAAGGGAACTGGTGC
	Reverse	AGCTGTCTATCATGGGTGTTG
USP11	Forward	GTATAACGTCCTGATGTACCGG
	Reverse	CTTTCTCATCCCCATCGTCC
USP12	Forward	TGTGATTGGGTGGAAGATGG
	Reverse	CAAAATAGTGCTCATTGACCGG
USP13	Forward	ATTCAGTGCTGTCAGACCC
	Reverse	CTTCTGCTTCCCTTCTCGTTAG
USP14	Forward	TGGCTTCAGCGCAGTATATTAC
	Reverse	TGTCCTTGTTCACCTTTCTCG
USP15	Forward	GGTCCTTGCCGCTATAATCTG
	Reverse	TTGGTCTTCAGATGCAGTGG
USP16	Forward	GATCTGAACCTCACTGTCTGG
	Reverse	TGTAATGCTGGCTTGTTTTCTG
USP18	Forward	TCATGCGATTCTCCATCAGG
	Reverse	AAAAGCTCATACTGCCCTCC
USP19	Forward	ATGTGAAGCCTGAGAACCTG
	Reverse	CTTAGCCAACTCTGAGGATAGC
USP20	Forward	ACCTTTGCCCTCACCTTG

	Reverse	TTCTCCGCAGCCAACATAG
USP22	Forward	GAGAGCAGGATGAATGGACAG
	Reverse	CGGATAAAGCTGGTGTAGTGG
USP24	Forward	CTTCTGCTCTTGATACCCACTG
	Reverse	CTGGTGCTGTTGCTTTGATG
USP25	Forward	AAGAGAAGCCAAAGAACCCC
	Reverse	TGAACCCATTGACCTGAAGTG
USP26	Forward	AGAATCTGTGAACAAGCCCC
	Reverse	TTGGAACCCAGTGCAAGTAG
USP29	Forward	AGACCTGGAAAATGGCTCTG
	Reverse	CCCTCCATCTTCATACATCACAG
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USP31	Forward	AATTCCTCTGCCCCACAC
	Reverse	TTTCCATAGACACTGCTTCCC
USP32	Forward	ACCTTCGCTATCAAACATCCC
	Reverse	CTCATTTTCCCCTAGCTCTTCC
USP33	Forward	GGATAGGAAATTAGGAACTCAGCC
	Reverse	TCAAATACGGCAACCAGAGG
USP34	Forward	GTCTACACGCCCTTATCTTCAG
	Reverse	CCTTGAGGACATCTATACAGGC
USP36	Forward	TCTCAAATGCCCAAGGAGTG
	Reverse	CTCTCCATTCCAAGACACAGG
USP37	Forward	GAAGAGAATAGGACATCAGGGC
	Reverse	GAGGGAGTCTGGCTTGAAAC
USP38	Forward	TCTCCCAGGTGACAAAAGTG
	Reverse	GCTTGCCAATGCTACAGAAG
USP39	Forward	GCTTCCCCATCCTGATCTG
	Reverse	GCTCCTTCTCGTCCTTGTAG
USP43	Forward	GTTCCTGACTCTCCCATCTTC
	Reverse	TCTTGAATGGTCCCTGCTTG
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	Reverse	TGTTTCTGGGCTTCTGTGAG
USP46	Forward	TTGTAAAGATGGCGGTGAGG
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USP47	Forward	CAGGTTTATAGGTCCGCTTCC
	Reverse	TGCTTGGTTTACTAGTCCCAC
USP48	Forward	TCTCAGACACGTAAACCCAAG
	Reverse	GAATTATCCCGATCTACCAGCTC
USP49	Forward	ATCTACGCTTGTGACCAGTG
	Reverse	GAATCTTTTAAGGTGCAGCCG
USP50	Forward	CTTCTCACTCCCCATTCCATC
	Reverse	GGTTTCACAAAAGGAGCAGTG
USP51	Forward	AGCCTGCCATAGTGTTTCTAC
	Reverse	TGGGTTCTGGGAATCGAATG
USP52	Forward	TGTTTCCGTCTCCCACTTTG
	Reverse	GCTCTGGATCTGCCGAATATC
USP54	Forward	TCATATCCCAAAGTCGCACAG
	Reverse	ACCTCTTCCTGCAATTCATCC
Mouse CSN5	Forward	GAATACCCTGAGTTCCTCTAGC
	Reverse	CTAAGCCCAACATGAAACTGC

Mouse CSN2	Forward	CATCCCTCACCCACTAATCATG
	Reverse	TCTTGGGCTTCCTGATTCATC
Mouse β-actin	Forward	ACCTTCTACAATGAGCTGCG
	Reverse	CTGGATGGCTACGTACATGG
CSN5 promoter p65 site mutant	Forward	CTTGAAGTAAACGAGATGACATGTTTATCTTCC
	Reverse	GGAAGATAAACATGTCATCTCGTTTACTTCAAG

Letters underlined indicate a mutation site.

Macrophage-conditioned medium preparation

Macrophage-conditioned medium was prepared as described previously (Wu et al., 2009). Primary human macrophages (Stemcell Technologies, Vancouver, BC, Canada) were seeded in RPMI 1640 medium with 10% heat-inactivated human serum (ThermoFisher Scientific) and 40 ng/ml human MCSF (BioLegend, San Diego, CA, USA). Cells were incubated for 7 days in the presence of MCSF and changed with fresh medium without MCSF on day 7. The cell culture continued for 24 hours and the cells were then exposed to lipopolysaccharide (100 ng/ml, Sigma-Aldrich) for 24 hours. The culture medium was collected, centrifuged, and stored in aliquots at -80°C.

Western blot analysis, immunocytochemistry, and immunoprecipitation

Western blot analysis was performed as described previously (Lim et al., 2008). Image acquisition and quantitation of band intensity were performed using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). For immunoprecipitation, the cells were lysed in buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, and 0.5% Nonidet P-40) and centrifuged at 16,000 × g for 30 minutes to remove debris. Cleared lysates were subjected to immunoprecipitation with antibodies. For immunocytochemistry, cells were fixed in 4% paraformaldehyde at room temperature for 15 minutes, permeabilized in 5% Triton X-100 for 5 minutes, and then stained using primary antibodies. The secondary antibodies used were

mouse Alexa Fluor 488 or 594 dye conjugate, or rabbit Alexa Fluor 488 or 594 dye conjugate (Life Technologies, Carlsbad, CA, USA). Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI blue; Life Technologies). After mounting, the cells were visualized using a multiphoton confocal laser-scanning microscope (Carl Zeiss, Thornwood, NY, USA).

COPS5 promoter and luciferase assay

The pEZX-*COPS5* Luc plasmid was purchased from GeneCopoeia. The mutant of p65 binding site of the *COPS5* promoter (*COPS5* encodes CSN5) was generated in the pEZX-*COPS5* Luc as a template by site-direct mutagenesis, and the primers are described in qRT-PCR. All constructs were confirmed by DNA sequencing. Cells were transfected using Lipofectamine 2000 (Life Technologies). pRL-TK (Promega, Madison, WI, USA) was co-transfected as an internal control for normalizing transfection efficiency. After transfection and experimental treatments, cells were lysed and luciferase activity was measured using the Dual Luciferase kit (Promega) according to the manufacturer's instructions. Protein expression from the luciferase assay was determined in the remaining cell lysate using Western blot analysis.

Tumor-infiltrating lymphocyte profile analysis by CyTOF or flow cytometry

Excised tumors were digested in collagenase/hyaluronidase (Stemcell Technologies) and DNase I (Sigma-Aldrich), and tumor-infiltrating lymphocytes (TILs) were enriched on a Ficoll gradient (Sigma-Aldrich). TILs were incubated with CD16/CD32 antibody (eBioscience, San Diego, CA, USA) to block FcγR binding for 10 minutes and then applied to Cy-TOF or flow cytometry. For CyTOF analysis, TILs were incubated with a mixture of metal-labeled antibodies (listed below) for 30 minutes at room temperature, washed twice, and incubated with Cell-ID Intercalator-^{103}Rh

(Fluidigm, San Francisco, CA, USA) overnight at 4 °C. The sample was analyzed with the CyTOF2 instrument (Fluidigm) in the Flow Cytometry and Cellular Imaging Core Facility at MD Anderson. For flow cytometry, cells were stained with CD3-PerCP (BioLegend), CD4-FITC (eBioscience), CD8-APC/Cy7 (BioLegend), IFNγ-Pacific Blue (BioLegend), CD45-BV510 (BioLegend), CD11b-APC/Cy7 (BioLegend), Gr-1-PE (BioLegend), or F4/80-APC (BioLegend) antibodies. Stained samples were analyzed using a BD FACSCanto II (BD Biosciences, San Jose, CA, USA) cytometer.

Antibodies used for Cy-TOF analysis (provided by Fluidigm):

Marker	**Clone**	**Label**
Gr-1	RB6-8C5	141Pr
CD11c	N418	142Nd
CD45	30-F11	147Sm
CD11b	M1/70	148Nd
CD19	6D5	149Sm
CD3e	145-2C11	152Sm
CD8a	53-6.7	168Er
TCRβ	H57-597	169Tm
NK1.1	PK136	170Er
CD4	RM4-5	172Yb
B220	RA3-6B2	176Yb

Plasmids

Human or mouse PD-L1 clone was obtained from the shRNA/ORF Core Facility at MD Anderson and cloned into pCDH lentiviral expression vectors to establish PD-L1-Flag or PD-L1-

Myc expression cell lines. The PD-L1 was also cloned into pCMV-HA mammalian cell expression vectors for transient transfection. To create a pGIPZ-shPD-L1/Flag-PD-L1 dual expression construct to knock down endogenous PD-L1 and reconstitute Flag-PD-L1 simultaneously (Lim et al., 2016), we first selected an shPD-L1 construct (shPD-L1 #5) that targets the 3′-UTR region of PD-L1 mRNA. Next, Flag-PD-L1 was cloned into pGIPZ-shPD-L1 (ThermoFisher Scientific, Pittsburgh, PA, USA), which expresses shRNA for endogenous PD-L1. All constructs were confirmed using enzyme digestion and DNA sequencing. Detailed information is available upon request.

TIL profile analysis

Mice receiving 1×10^5 4T1 or EMT6 cells were treated with LPS, TNFα antibody, or clodronate liposome as described in the previous section. Excised tumors were digested in collagenase/hyaluronidase (Stemcell Technologies, Vancouver, BC, Canada) and DNase I (Sigma), and lymphocytes were enriched on a Ficoll gradient (Sigma). T cells were isolated using the Dynabeads untouched mouse T cell kit (Invitrogen, Carlsbad, CA) and stained using CD3-PerCP (BioLegend, San Diego, CA, USA), CD4-FITC (eBioscience, San Diego, CA, USA), CD8-APC/Cy7 (BioLegend), CD45.1-PE (BioLegend), and IFNγ-Pacific Blue antibodies (BioLegend). Stained samples were analyzed by FACS using a BD FACSCanto II (BD Biosciences) cytometer.

PD-L1 and PD-1 interaction assay and T cell-mediated tumor cell killing assay

To measure PD-1 and PD-L1 protein interaction, we seeded cells in 96-well plates and then incubated them with recombinant human PD-1 Fc protein (R&D Systems, Minneapolis, MN,

USA) and anti-human Alexa Fluor 488 dye conjugate (Life Technologies). Every hour, green fluorescent signal was measured and quantified by IncuCyte Zoom (Essen BioScience, Inc., Ann Arbor, MI, USA). The T cell-mediated tumor cell killing assay was performed as described previously (Lim et al., 2016). To analyze the killing of tumor cells by T cell inactivation, we co-cultured nuclear restricted red fluorescent protein-expressing tumor cells with activated primary human T cells or peripheral blood mononuclear cells (Stemcell Technologies) in the presence of Caspase 3/7 substrate (Essen Bioscience) in 96-well plates. T cells were activated with CD3 antibody (100 ng/ml) and IL-2 (10 ng/ml). Every 2 hours, red fluorescent protein and green fluorescent (NucView 488 Caspase 3/7 substrate) signal were measured and quantified by IncuCyte Zoom (Essen BioScience). Green fluorescent cells were counted as dead cells. Alternatively, to visualize the survived tumor cells at the end point, after 4 days co-culture of tumor cells and T cells in 12-well plates, wells were washed with PBS twice to remove T cells and then the survived tumor cells were fixed and stained with crystal violet solution. The dried plates were scanned and quantified the intensity.

Immunofluorescence

Tumor masses were frozen in an OCT block immediately after extraction. Cryostat sections of 5-μm thickness were attached to saline-coated slides. Cryostat sections were fixed with 4% paraformaldehyde for 15 min at room temperature and blocked with blocking solution (5% bovine serum albumin, 2% donkey serum, and 0.1M PBS) at room temperature for 30 min. Samples were stained with primary antibodies against F4/80 (1:100; Abcam, Cambridge, MA, USA) and Granzyme B (1:300; R&D Systems) overnight at 4 °C, followed by donkey anti-rat Alexa 594 and donkey anti-goat Alexa 488 (Invitrogen, 1:3,000) secondary antibodies at room

temperature for 1 hour. Nuclear staining was performed with Hoechst 33342 (Molecular Probes). A confocal microscope (Carl Zeiss, LSM700) was used for image analysis.

Immunohistochemical staining of human breast tumor tissue samples

Human breast tumor tissue specimens were obtained following the guidelines approved by the Institutional Review Board at MD Anderson, and written informed consent was obtained from patients in all cases. Immunohistochemical staining was performed as described previously (Li, 2016Li, 2016). Briefly, tissue specimens were incubated with antibodies against p-p65, PD-L1, CSN5, or granzyme B and a biotin-conjugated secondary antibody and then incubated with an avidin-biotin-peroxidase complex. Visualization was performed using amino-ethylcarbazole chromogen. According to histologic scoring, the intensity of staining was ranked into 1 of 4 groups: high (score 3), medium (score 2), low (score 1), and negative (score 0).

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