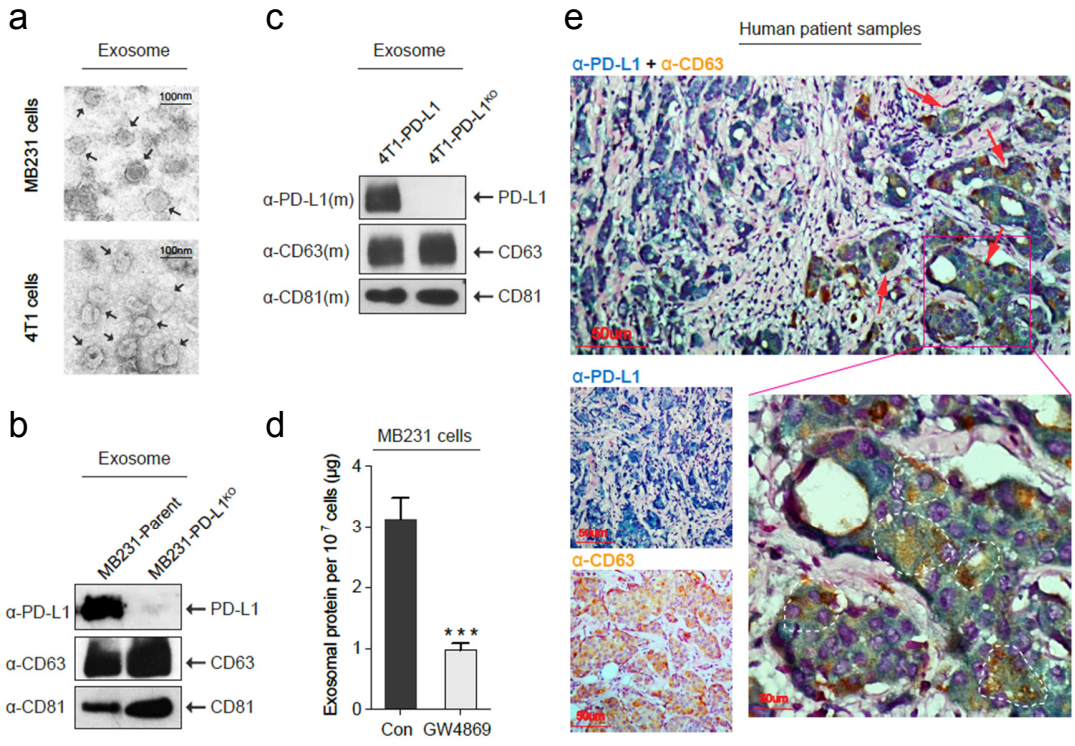
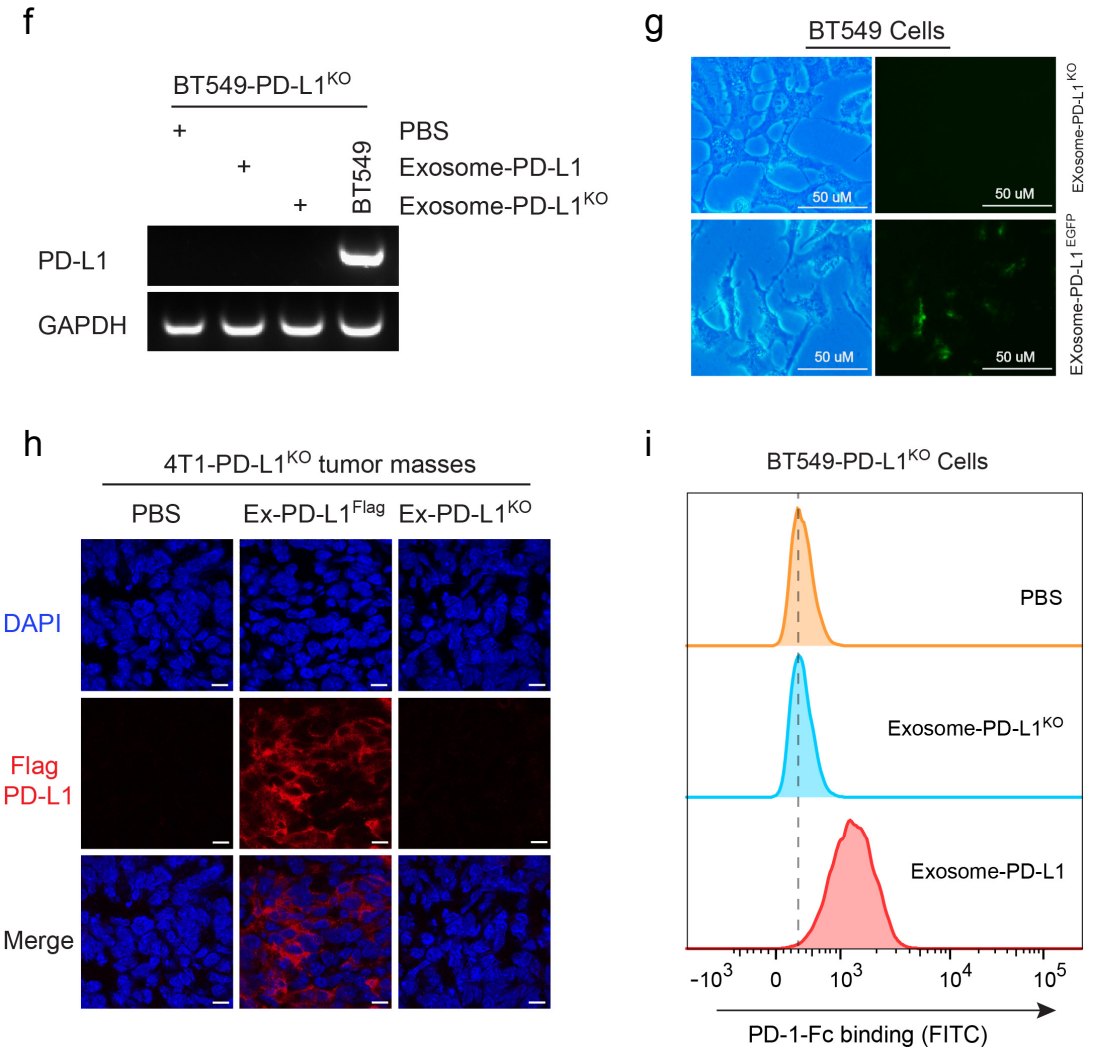
Supplementary Information





Supplementary information, Figure S1. PD-L1 existed in exosomes and can be transported to other cells.

a Transmission electron microscopy of exosomes isolated from the media of human breast cancer cells MB231 or mouse mammary tumor cells 4T1.

b, **c** Analysis of PD-L1 in exosomes isolated from the media of MB231 parental and MB231-PD-L1^{KO} cells (**b**), 4T1-PD-L1 and 4T1-PD-L1^{KO} cells (**c**). Exosomes were confirmed by Western blot with exosome makers CD63 and CD81.

d The total amounts of proteins in the exosomes purified from MB231 cells treat with exosome secretion inhibitor GW4869 (10 μM) or DMSO (control) were

quantified by Bradford assay. Data showing the amounts of exosomal proteins per 10 million secreting cells.

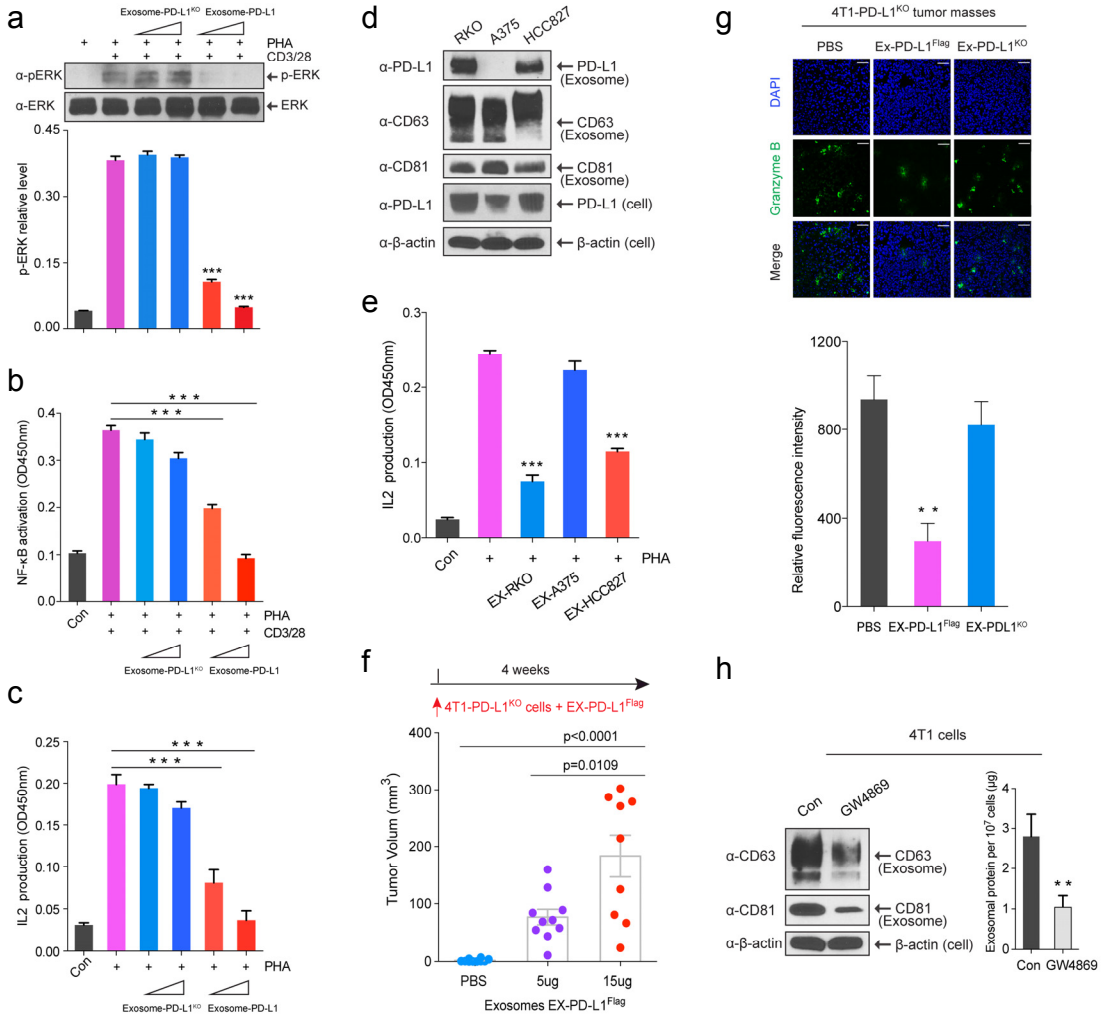
e IHC double staining of tumor specimens from breast cancer patients. Blue: PD-L1 expression, brown: CD63 expression, green/brown: co-localization of PD-L1 and CD63 (red arrows and dashed circles).

f BT-549-PD-L1^{KO} cells were treated with exosomes isolated from exosome-PD-L1^{KO}, exosome-PD-L1, or PBS for 24 hours and then subjected to RT-PCR to detect PD-L1 mRNA. BT549 cells were used as positive control.

g Exosome-PD-L1^{EGFP} or exosome-PD-L1^{KO} were added into BT549 cells. After incubation of 24 hours, cells were washed for 4 times with PBS and then subjected to EGFP detection using fluorescence microscopy.

h Immunofluorescence staining of the protein expression pattern of PD-L1 (Flag, red) in 4T1-PD-L1^{KO} tumors (with co-injection of EX-PD-L1^{Flag}, EX-PD-L1^{KO} or PBS, and growth for 5 days). Scale bar, 10 μm.

i Exosome-PD-L1, exosome-PD-L1^{KO}, or PBS was added into BT549-PD-L1^{KO} cells. After 24-hour incubation, cells were washed with PBS 4 times and subjected to flow cytometric analysis of cell surface PD-L1 using PD-1-Fc antibody.



Supplementary information, Figure S2. PD-L1-containing exosomes inhibited T cell functions, promoted tumor growth.

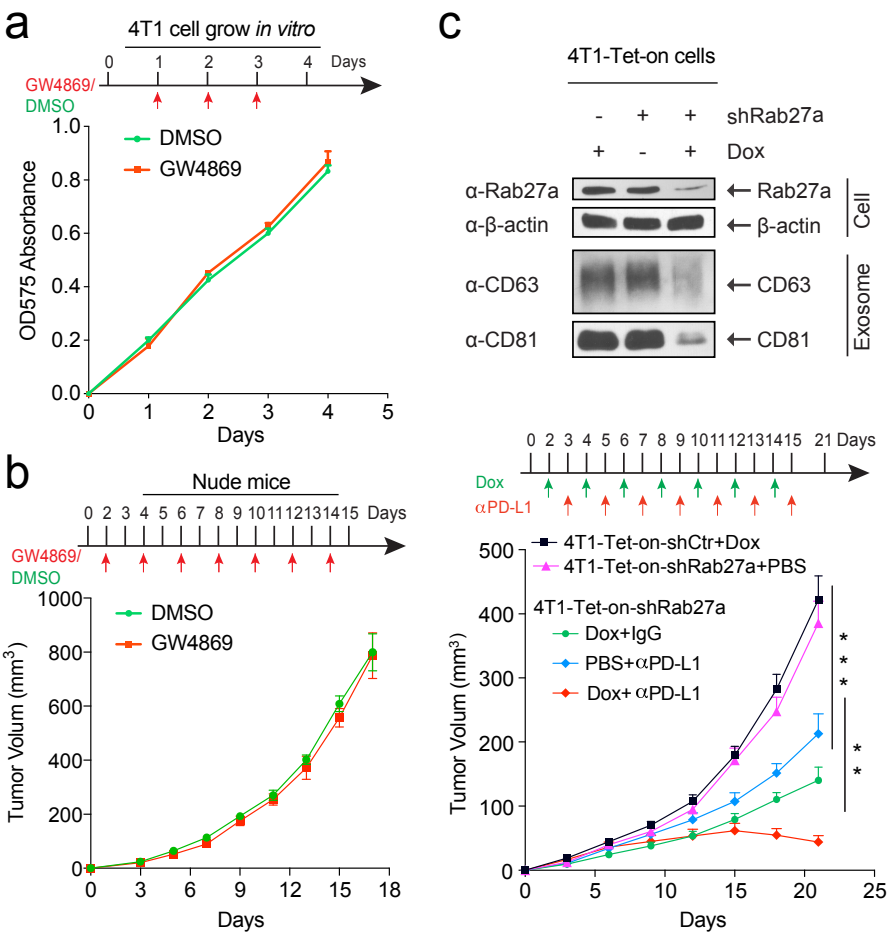
a-**c** Peripheral blood mononuclear cells (PBMC) were treated as indicated. ERK phosphorylation (**a**) was determined by Western blotting (quantification of p-ERK expression level showing at the bottom); NF-κB activation (**b**) was detected using TransAM NF-κB p65 Transcription factor assay Kit; and IL-2 secretion (**c**) measured using Human IL-2 Ready-SET-Go! ELISA kit. Data represent mean ± SD of three independent experiments. ***$P < 0.001$, Student's *t*-test.

d, **e** Western blot analysis of PD-L1 and exosome markers CD63/CD81 (**d**) in exosomes isolated from colon cancer cell line RKO, melanoma cell line A375, and lung cancer cell line HCC827. Exosomes were incubated with PBMC with 0.1 μg/mL PHA. After 3-day incubation, IL-2 secretion (**e**) was measured using the Human IL-2 Ready-SET-Go! ELISA kit. Data represent mean ± SD of three independent experiments. ***P < 0.001, Student's t-test.

f Tumor growth of 4T1-PD-L1^{KO} cells in BALB/c mice injected with increasing amounts of EX-PD-L1^{Flag}. Tumor volume was measured after 4 weeks (n = 10 mice per group). Data represent mean ± SEM. P values as indicated, Student's t-test.

g Top, immunofluorescence staining of granzyme B expression in 4T1-PD-L1^{KO} tumors with co-injection of EX-PD-L1^{Falg}, EX-PD-L1^{KO}, or PBS and growth for 5 days. Scale bar, 50 μm. Bottom, relative fluorescence intensity quantified using ImageJ software. Data represent mean ± SEM. **P < 0.01, Student's t-test.

h Western blot analysis of exosome markers CD63/CD81 in exosomes isolated from 4T1 cells with or without GW4869 (10 μM) treatment for 24 hours. The total amounts of proteins in the exosomes were quantified by Bradford assay. Data showing the amounts of exosomal proteins per 10 million cells. **P < 0.01, Student's t-test.



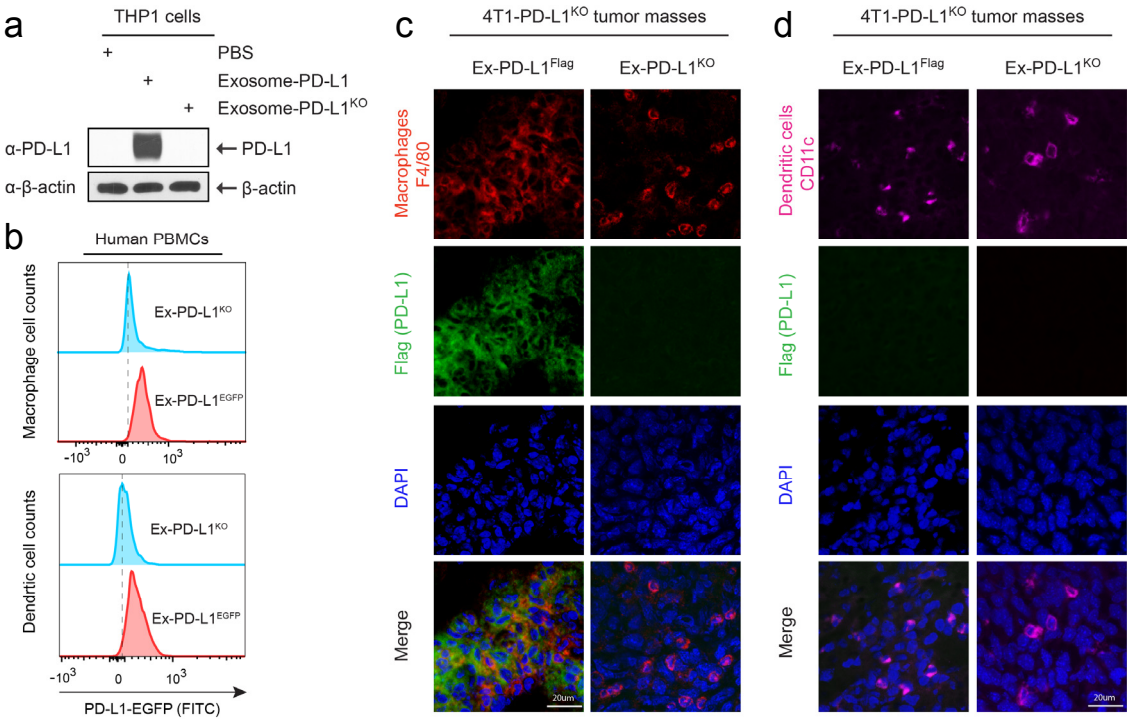
Supplementary information, Figure S3. Inhibition of exosomes secretion augmented anti-PD-L1 therapeutic efficacy in 4T1 tumor growth suppression.

a Growth curve of 4T1 cells treated with GW4869 (10 μM) or DMSO control as indicated. Data represent mean ± SD of three independent experiments.

b Tumor growth of 4T1 cells in nude mice treated with GW4869 (2.5 μg/g) or DMSO control as indicated. Tumor volume was measured at the indicated time points (n = 5 mice per group). Data represent mean ± SEM.

c Top: Western blot analysis of Rab27a expression in 4T1-Tet-on-shRab27a inducible cells with or without doxycycline (1μg/mL) treatment for 2 days as indicated, and exosome markers CD63/CD81 expression in exosomes isolated from those cells. Bottom: Tumor growth of 4T1-Tet-on-shCtr or 4T1-Tet-on-

shRab27a inducible cells in BALB/c mice treated with doxycycline (50 μg/g) or PBS and/or PD-L1 antibody as indicated. Tumor volume was measured at the indicated time points (n = 8 mice per group). Data represent mean ± SEM. **P < 0.01, ***P < 0.001 or as indicated, Student's t-test.

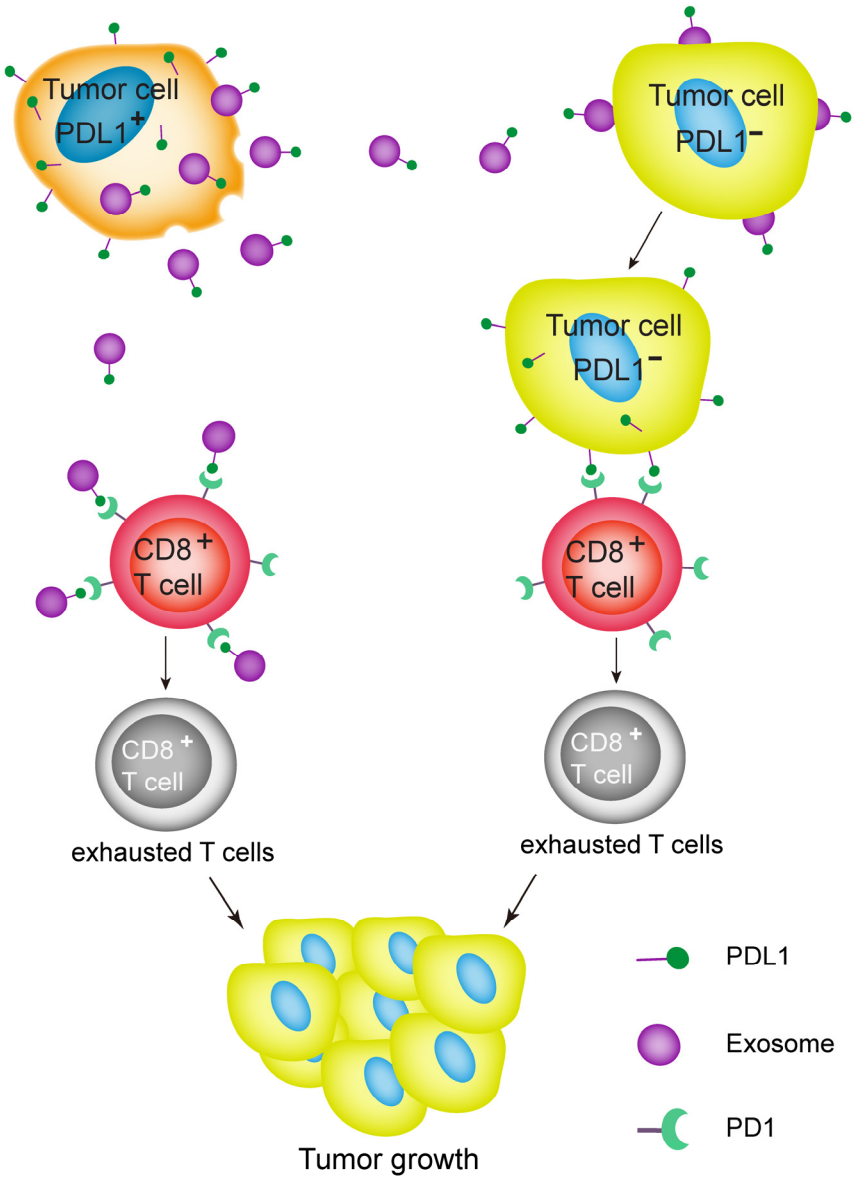


Supplementary information, Figure S4. Exosomes transported PD-L1 to macrophages and dendritic cells *in vitro* or *in vivo*.

a Exosome-PD-L1, exosome-PD-L1^{KO}, or PBS was added to THP1 cells. After 24-hour incubation, cells were washed with PBS 4 times, lysed in lysis buffer, and subjected to Western blotting using the indicated antibodies.

b Exosome-PD-L1^{EGFP} or exosome-PD-L1^{KO} was added into human PBMCs. After 24-hour incubation, cells were washed with PBS 4 times and subjected to flow cytometric analysis of PD-L1^{EGFP} in Macrophages gated on CD11b^{+} and DCs gated on CD11c^{+}.

c, **d** Immunofluorescence staining of the protein expression pattern of PD-L1 (Flag, green) in macrophages (**c**, F4/80, red) or DCs (**d**, CD11c, purple) in 4T1-PD-L1^{KO} tumors (with co-injection of EX-PD-L1^{Flag} or EX-PD-L1^{KO}, and growth for 5 days).



Supplementary information, Figure S5. A proposed working model showing PD-L1 in exosomes from cancer cells.

Exosomal PD-L1 actively exhausts T cells by rendering PD-L1-negative cancer cells PD-L1 positive or by direct binding to PD-1 on T cells. The engagement of PD-L1 and PD-1 results in immunosuppression in the tumor microenvironment in favor of tumor growth.

Supplementary Materials and Methods

Cell culture. All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and have been independently validated using STR DNA fingerprinting at MD Anderson, and tests for mycoplasma contamination were negative. Cells were grown in DMEM medium supplemented with 10% fetal bovine serum. MDA-MB-231-PD-L1, 4T1-PD-L1, MDA-MB-231-PD-L1^{KO}, BT549-PD-L1^{KO}, and 4T1-PD-L1^{KO} cells have been previously described[1]. MDA-MB-231-PD-L1-EGFP stable cell clones were selected using puromycin (InvivoGen, San Diego, CA, USA). Peripheral blood mononuclear cells (PBMCs) were purchased from STEMCELL Technologies (Vancouver, BC, Canada).

Exosome isolation and purification. Exosomes were collected from cell culture media after sequential ultracentrifugation as described previously[2]. In brief, cells were cultured for 72 hours and supernatant collected by centrifugation at 300× *g* for 10 min. 2,000× *g* for 10 min, and 10,000*g* for 30 min, and then ultracentrifuged at 100,000× *g* to pellet the exosomes. The pellets were washed in a large volume of PBS to eliminate contaminating proteins and centrifuged one last time at 100,000× *g*. The final pellet containing exosomes were re-suspended in PBS. The total exosomal protein concentrations were determined by using the BCA Protein Assay kit (Thermo Fisher Scientific, Santa Clara, CA).

Transmission electron microscopy. Exosome samples were placed on 100-mesh carbon coated, formvar-coated copper grids treated with poly-l-lysine for approximately 30 min. Samples were then negatively stained with Millipore-filtered aqueous 1% uranyl acetate for 1 min. Stain was blotted dry from the grids with filter paper and samples were allowed to dry. Samples were then examined in a JEM 1010 transmission electron microscope (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 80 Kv. Digital images were obtained using the AMT Imaging System (Advanced Microscopy Techniques Corp., Danvers, MA). All

processes were performed at the High Resolution Electron Microscopy Facility at MD Anderson Cancer Center.

Detection of mRNA. Total RNA was prepared with Trizol reagent (Invitrogen, Carlsbad, CA). The cDNA was synthesized using SuperScript™ First-Strand Synthesis System (Invitrogen). PD-L1 mRNA expression was analyzed using the following primer set:

Forward: 5′-CATACAACAAAATCAACCAAAG-3′

Reverse: 5′-TGGAGGATGTGCCAGAGGTAG-3′

Immunofluorescence. For immunofluorescence staining of cultured MDA-MB-231 cells, cells were seeded and allowed to attach overnight. Adherent cells were fixed with 4% paraformaldehyde for 20 min and blocked with 2% BSA and 0.5% Triton X-100 in PBS, cells were stained with primary antibodies against PD-L1 (1:100; E1L3N, Cell Signaling Technology, Danvers, MA) and CD63 (1:100; 353013, BioLegend, San Diego, CA) overnight at 4°C, followed by goat anti-rabbit Texas Red (1: 1,000; Invitrogen) and goat anti-mouse Alexa 488 (1: 1,000; Invitrogen) secondary antibodies incubation at room temperature for 1 hour. For immunofluorescence staining of tumor tissues, 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 (2% BSA in PBS) at room temperature for 30 min. Samples were stained with primary antibodies against Flag (1:100; 14793S, Cell Signaling Technology, Danvers, MA), mGranzyme B (1:100; AF1865, R&D Systems), mF4/80 (1:100; 123121, BioLegend, San Diego, CA) or mCD11c (1:100; 117314, BioLegend, San Diego, CA) as indicated overnight at 4°C, followed by goat anti-rabbit FITC (1:1,000; Invitrogen, Carlsbad, CA) and donkey anti-goat Alexa 488 (1:1,000; Invitrogen) secondary antibodies incubation at room temperature for 1 hour. Nuclear staining was performed with VECTASHIELD mounting medium with

DAPI (Vector Laboratories, Burlingame, CA). A confocal microscope (LSM700, Carl Zeiss, Oberkochen, Germany) 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 following the manufacture's protocol (MULTIVIEW® mouse-HRP/rabbit-AP IHC kit; Enzo Biochem, Inc., Farmingdale, NY). Briefly, tissue specimens were incubated with primary antibodies against PD-L1 (ab205921, Abcam, Cambridge, MA), CD63 (353013, BioLegend) overnight at 4°C followed by HRP/AP-conjugated secondary antibody incubation at room temperature for 1 hour. Visualization was performed using DAB and green chromogen. For the human breast cancer tissue microarray, the co-localizations of PD-L1 and CD63 were represented by percentage from 0%-100%.

Flow cytometry analysis

Exosomal PD-L1 and its binding with PD-1-Fc. Exosome-PD-L1, exosome-PD-L1^{KO} or PBS was added into BT549-PD-L1^{KO} cells. After 24-hour incubation, cells were collected and washed with PBS 4 times followed by incubation with PD-1-Fc protein (1086-PD-050, R&D Systems) overnight at 4°C and then goat anti-human IgG Fc Alexa 488 secondary antibody at 4°C for 1 hour. Cells were subjected to FACS analysis using the BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA)

PD-L1^{EGFP} in macrophages or DCs. Exosome-PD-L1^{EGFP} or exosome-PD-L1^{KO} was added into human PBMCs. After 24-hour incubation, cells were washed with PBS 4 times and subjected to FACS analysis of PD-L1^{EGFP} in Macrophages gated on CD11b^{+} (1:100; 301309, BioLegend, San Diego, CA) and DCs gated on CD11c^{+} (1:100; 337217, BioLegend, San Diego, CA) using the BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA)

T-cell-mediated tumor cell-killing assay. T cells (PBMCs) were activated with CD3/CD28 antibody (100 ng/mL; 10971, STEMCELL Technologies, Vancouver, BC, Canada) and IL-2 (10 ng/mL; CYT-209, ProSpec, Ness-Ziona, Israel). After 5 days of co-culture of tumor cells and T cells with or without exosomes in 24-well plates, wells were washed with PBS 3 times to remove T cells. The survived tumor cells were fixed and stained with crystal violet solution. The dried plates were scanned, stains solubilized in 100 μL 33% glacial acetic acid, and shaken until color is uniform. The absorbance was measured at 570 nm using a spectrometer (Synergy NEO, BioTek, Winooski, VT).

T cell NF-κB activation and ERK phosphorylation assay. PBMCs were cultured in RPMI containing 10% FBS and PHA (L-1110, VECTOR, Burlingame, CA) at 0.1 μg/mL as indicated for 3 days, followed by CD3/CD28 treatment with increasing amounts of exosome-PD-L1 or exosome-PD-L1^{KO} for 30 min. Cells were then collected and lysed. NF-κB transcription activities were determined following the manufacturer's instructions (40096; TransAM NF-κB p65 Transcription factor assay Kit, Active Motif, Carlsbad, CA). ERK phosphorylation was determined by Western blotting.

IL-2 ELISA assay. PBMCs were cultured in RPMI containing 10% FBS and PHA at 0.1 μg/mL as indicated with increasing amounts of exosome-PD-L1 or exosome-PD-L1^{KO} for 3 days. IL-2 production was determined by collecting cells supernatant and incubating on IL-2 ELISA plates following the manufacturer's instructions (88-7025; Human IL-2 Ready-SET-Go! ELISA kit, Thermo Fisher Scientific, Santa Clara, CA)

Animal Studies. Female BALB/c mice or *Foxn1^{nu}* nude mice (6–8-weeks old) were purchased from Jackson Laboratory). All animal procedures were

conducted under the guidelines approved by the IACUC at the MD Anderson Cancer Center. 4T1 cells (5×10^4 cells) in 50 µl of medium with exosomes as described mixed with 50 µl of Matrigel (BD) were injected into the BALB/c mice mammary fat fad. 4T1 cells (5×10^4 cells) in 50 µl of medium mixed with 50 µL of Matrigel were injected into the BALB/c mice or nude mice mammary fat fad, and after 2 days, mice were intraperitoneally injected with GW4869 (2.5 µg/g, D1692, Sigma-Aldrich, St Louis, MO) and/or 100 µg anti-PD-L1 antibody (BE0101-A, Bio X Cell, West Lebanon, NH) as indicated. 4T1-Tet-on-shCtr/shRab27a cells (5×10^4 cells) in 50 µL of medium mixed with 50 µL of Matrigel were injected into the BALB/c mice mammary fat fad, and after 2 days, mice were intraperitoneally injected with doxycycline (50µg/g, D9891, Sigma-Aldrich, St Louis, MO) and/or 100 µg anti-PD-L1 antibody (BE0101-A, Bio X Cell, West Lebanon, NH) as indicated. Tumors were measured every 3 days with a caliper and tumors volume calculated using the formula: $\pi/6 \times \text{length} \times \text{width}^2$.

Supplementary References

1 Lim, S. O. *et al.* Deubiquitination and Stabilization of PD-L1 by CSN5. *Cancer cell* **30**, 925-939, doi:10.1016/j.ccell.2016.10.010 (2016).

2 Thery, C., Amigorena, S., Raposo, G. & Clayton, A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Current protocols in cell biology* **Chapter 3**, Unit 3 22, doi:10.1002/0471143030.cb0322s30 (2006).