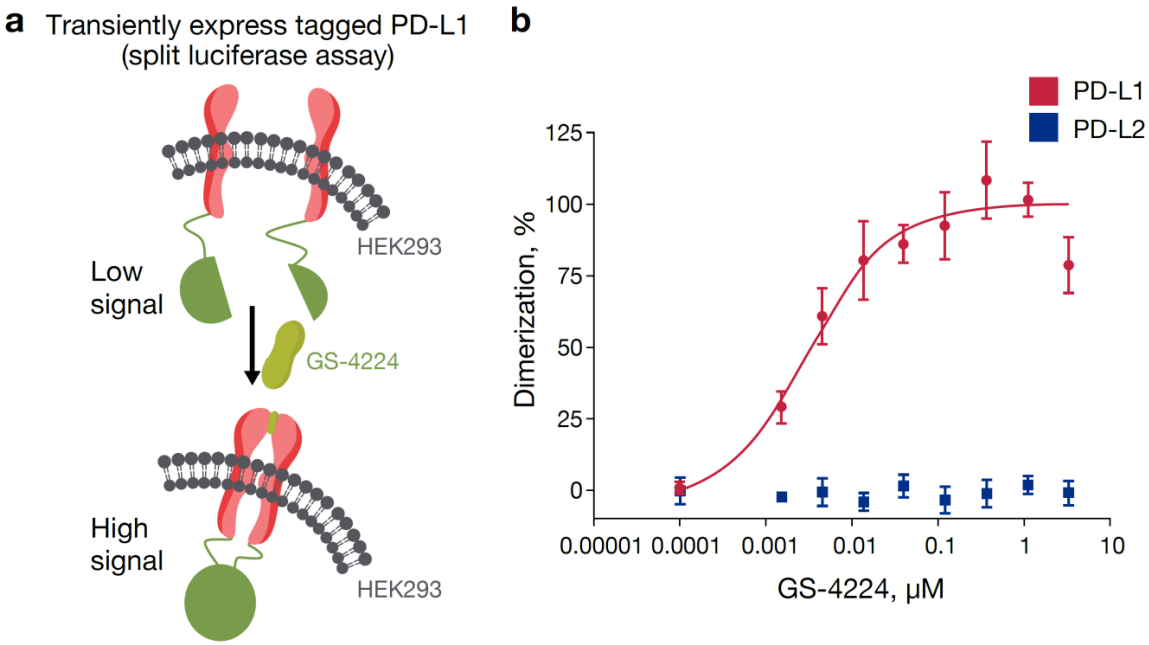
Supplemental Figures and Tables

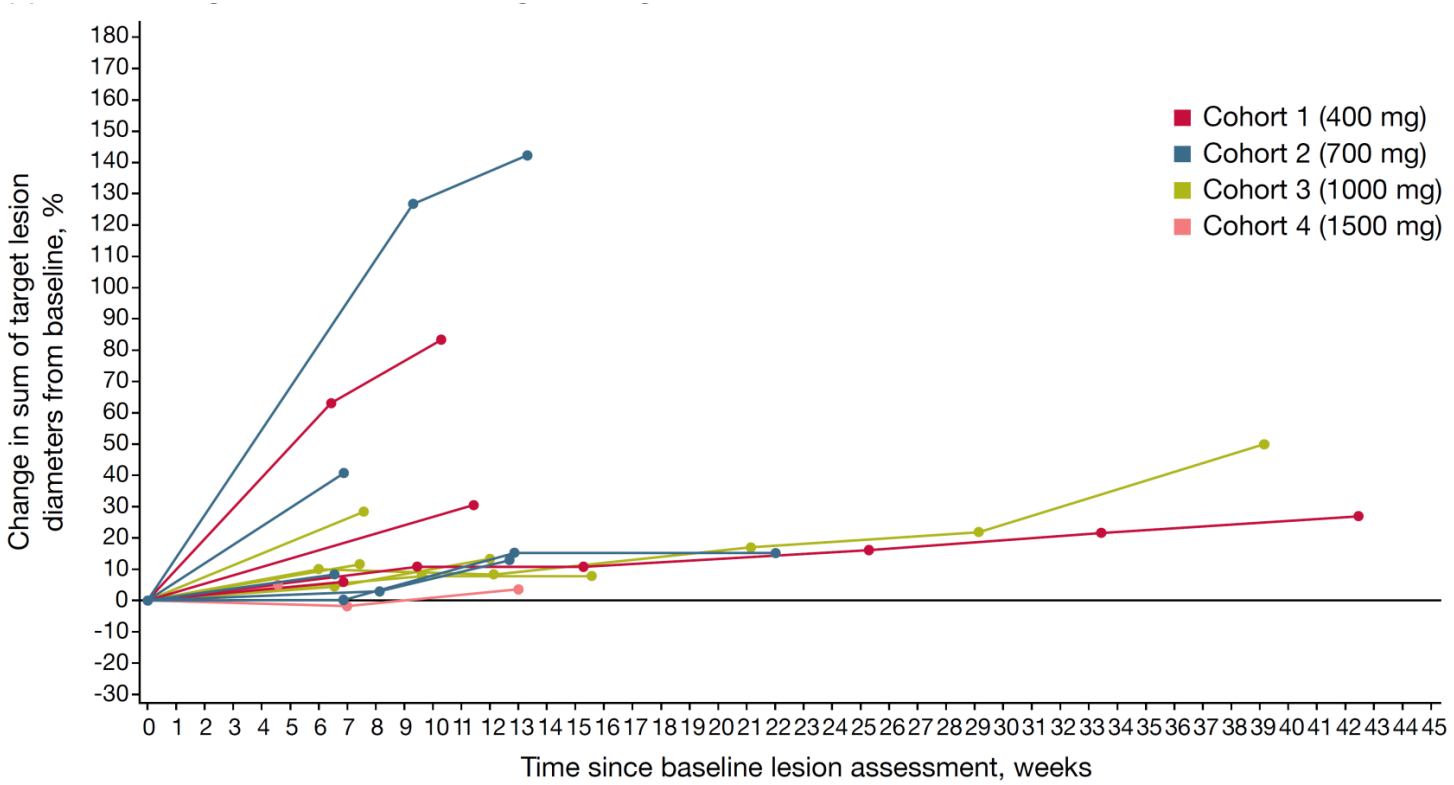
Supplemental Figure 1. GS-4224 Specifically Dimerizes PD-L1 on the Cell Surface



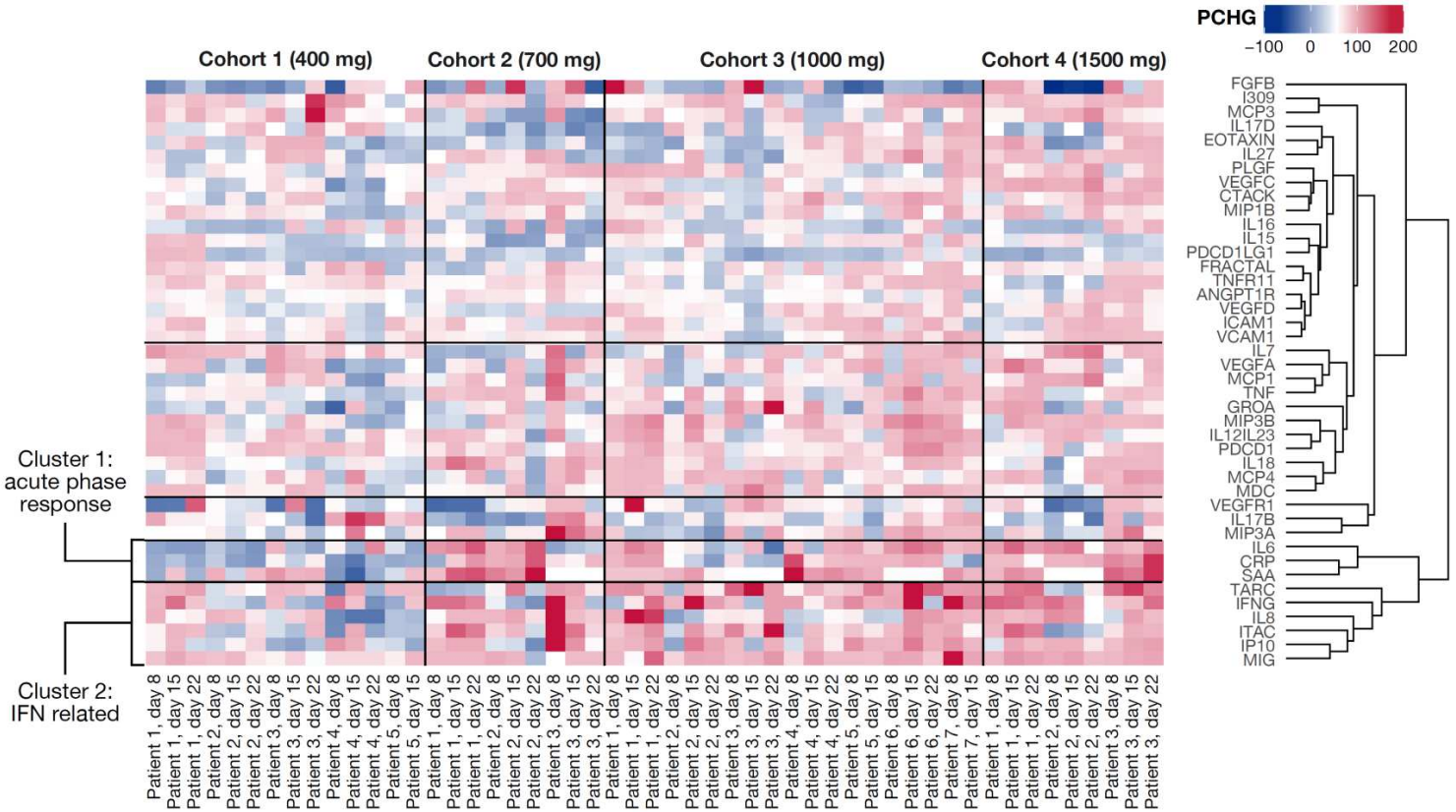
(a) Schematic overview of the split luciferase assay and (b) percent dimerization by GS-4224 dose. PD-L1, programmed death ligand 1; PD-L2, programmed death ligand 2.

(a) Schematic representation of GS-4224 induced PD-L1 dimerization in context of a NanoBiT assay designed to measure cell-bound PD-L1 dimerization. In the presence of increasing concentrations of GS-4224, (b) PD-L1 (red) but not PD-L2 (blue) dimerization was observed. Data is plotted as a mean of four replicates per GS-4224 concentration ± SD.

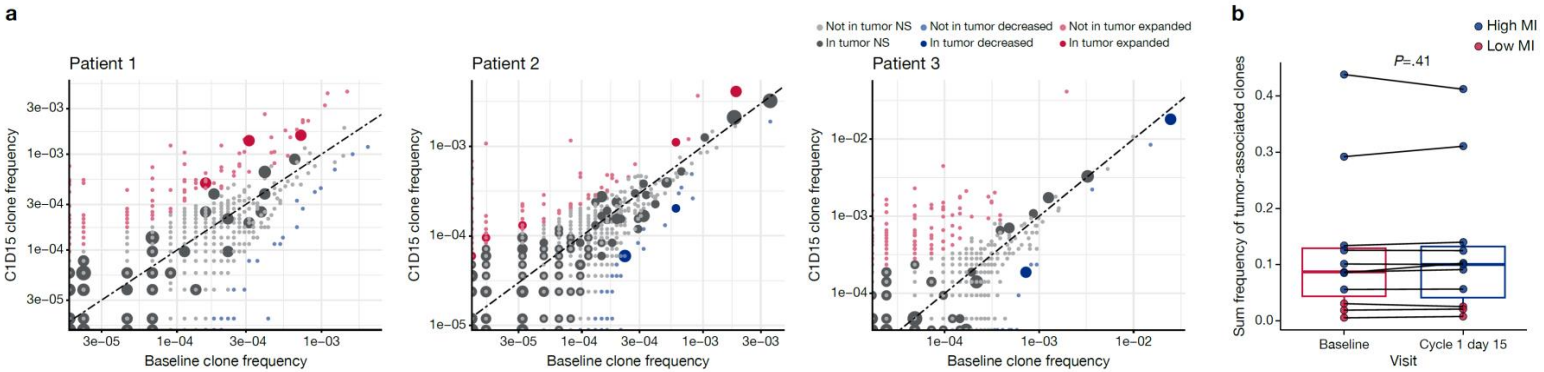
Supplemental Figure 2. Percent Change in Target Lesion Diameter From Baseline



Supplemental Figure 3. Immune Response Following Treatment With GS-4224



Supplemental Figure 4. Clone Frequency in (A) Select Tumor Samples and (B) From Baseline to Cycle 1 Day 15



C, cycle; D, day; MI, Morisita index; NS, not significant. Dot size indicates clone frequency in the tumor.

Supplemental Table 1. PD-L1 Status and Tumor Mutational Burden by Tumor Type and Dose Cohort

Dose level	Diagnosis	Prior anti-PD-1/PD-L1	PD-L1 status	TMB (mut/MB)
400 mg	Uterine leiomyosarcoma	No	NA	3.0
	Mesothelioma	No	Negative	0.7
	Gastroesophageal cancer	No	Negative	1.9
	Mesothelioma	No	Positive	NA
	Type II papillary renal cell carcinoma	No	Positive	5.7
700 mg	Pancreatic cancer	No	NA	NA
	Esophageal adenocarcinoma	No	NA	NA
	Renal cell carcinoma	No	Positive	16.2
1000 mg	Triple-negative breast cancer	No	Negative	7.0
	Castrate-resistant prostate cancer	No	Negative	0.3
	Renal cell carcinoma	No	NA	NA
	Squamous cell lung cancer	No	Positive	NA
	Urothelial cancer	No	NA	NA
	Endometrial cancer	No	Positive	1.1
	Anal squamous cell carcinoma	Nivolumab	Positive	NA
1500 mg	Hepatocellular carcinoma	No	Positive	NA
	Non-small cell lung cancer	Durvalumab	Positive	1.4
	Urothelial cancer	No	Positive	7.6

mut, mutations; NA, not available; PD-1, programmed cell death 1; PD-L1, programmed death ligand 1; TMB, tumor mutation burden. PD-L1 status was determined by local results reported in the case report form. Where local results were not available, PD-L1 was assessed retrospectively using the 22C3 PD-L1 IHC assay and a combined proportion score ≥ was considered positive for descriptive purposes.

Supplemental Methods

Measurement of cell surface PD-L1 dimerization using a split luciferase assay

To assess surface PD-L1 dimerization, a split luciferase assay called NanoLuc Binary Technology (NanoBiT) was established. Large BiT (LgBiT; 17.6 kDa) and Small BiT (SmBiT; 11 amino acids) luciferase subunits are fused to full length human PD-L1 or PD-L2. When the two subunits are expressed and brought into close proximity to form the functional enzyme, a luminescent signal can be detected using a cell permeable substrate. Expi293 cells (Thermo Fisher Scientific) were cultured in Expi293 expression medium. Transfection of Expi293 cells were performed using ExpiFectamine 293 Reagent following manufacturer's protocol at a density of 2.5 x 10^6 cells/mL. Expression constructs of full length human PD-L1-LgBiT (pRSG305) and human PDL1-Small Bit (pRSG306) were generated by Promega Corp (Madison, WI). Human PDL1-LgBit, hPDL1-SmBit and Transfection Carrier DNA (Promega) were first diluted in OptiMEM medium (Thermo Fisher Scientific) to 0.5 mg/mL. Human PDL1-LgBit and hPDL1-SmBit DNA were serially diluted with the 0.5 mg/mL TCD as diluent to concentrations of 50 and 1.56 µg/mL, respectively. For every 5 mL of Expi293 cells, 5 µL each of the diluted hPDL1-LgBit and hPDL1-SmBit were combined to form a transfection complex with ExpiFectamine 293 Reagent before addition to cells. At 23 to 24 hours post-transfection, cells were counted in a Vi-CELL Counter (Beckman Coulter). Transfected cells were centrifuged in a Beckman Coulter table top centrifuge at 500g for 5 minutes. Media was removed and cells were resuspended in OptiMEM medium at a density of 0.5 x 10^6 cells/mL. Cells were plated at 100 µL/well in the 96-well white opaque assay plate (Corning Costar).

To assess PD-L1 dimerization, varying concentrations of GS-4224 in triplicate were added to cells using a D300e Digital Dispenser (Tecan) at a final DMSO concentration of 0.1% and incubated for 30 minutes at 37°C in a humidified incubator containing 5% CO_2. After incubation, 25 µL of 20X dilution of Nano-Glo Live Cell Substrate (LCS) using Nano-Glo LCS Dilution Buffer (Promega) was added to each well. Luciferase activity was measured using a SpectraMax i3x Microplate Reader (Molecular Devices).

In vitro PD-L1 occupancy assay

Human lung adenocarcinoma cell line

Green fluorescent protein (GFP-expressing A549 lung adenocarcinoma cells (Creative Biogene, Shirley, NY) were harvested with trypsin and resuspended in Dulbecco's Modification of Eagle's Medium(DMEM) with 10% fetal bovine serum (FBS), non-essential amino acids, 100 U penicillin/streptomycin at 5 x 10^5 cells/mL. Fifty microliters of the cell preparation (25,000 cells/well) was then dispensed in each well of the 384-well assay plate along with a titration of GS-4224. The cell-compound mixture was supplemented with either 60 pg/mL IFN-γ (low PD-L1 induction) or 20 ng/mL IFN-γ (high PD-L1 induction), and were incubated for 48 hours at 37°C in a humidified incubator containing 5% CO_2. GS-696882, atezolizumab analogue with mouse IgG1 Fc produced in house, was added to each well already containing 50 µL of cells and incubated for 15 minutes at 4°C. Three µg/mL APC-conjugated α-mouse IgG1 was then added to the cells after wash followed by 20 minutes of incubation at 4°C. Fluorescence levels of PD-L1 were measured on an IQue Screener Plus flow cytometer (Intellicyt). Quantibrite PE beads (Becton Dickinson, Franklin Lakes, NJ) were reconstituted and acquired on an LSRFortessa X-20 for PD-L1 quantification according to the manufacturer instructions.

Primary human $CD3^+$ T cells

Peripheral blood mononuclear cells (PBMC) samples were either left unstimulated or stimulated for 48 hours at 37°C, 5.0% CO_2 with 1:40 ImmunoCult Human CD3/CD28 T Cell Activator (Stemcell Technologies, Vancouver, Canada), and 100 U/mL IL-2 (Sigma, St. Louis, MO) at a cell density of 10^6 cell/mL. Cells were then added accordingly to a 96-well flat-bottom plate at a density of 1 x 10^5 cell per well. For flow analysis, cells were incubated with Human TruStain Fc Receptor (FcX) solution (Biolegend, San Diego, CA) for 5 minutes at room temperature and then stained with CD2 (RPA-2.10) PerCP-Cy5.5 (Biolegend, San Diego, CA), CD25 (BC96) PE-Cy7

(Thermo Fisher Scientific, Waltham, MA), Fixable Viability Dye eFluor 780 (Thermo Fisher Scientific, Waltham, MA), and custom PD-L1 (atezolizumab, Genentech) PE (Southern Biotech, Birmingham, AL) for 30 minutes at room temperature in a final volume of 200 µL per well. Samples were transferred after washing to a 96-well, U-bottom plate and acquired on an LSRFortessa X-20 (Becton Dickinson, Franklin Lakes, NJ). Quantibrite PE beads (Becton Dickinson, Franklin Lakes, NJ) were reconstituted and acquired on an LSRFortessa X-20 for PD-L1 quantification according to the manufacturer instructions.

Peripheral human whole blood

Peripheral human whole blood samples were collected in sodium heparin vacutainers (Becton Dickinson, Franklin Lakes, NJ). Samples were aliquoted at 100 µL per test and exposed to a titration of GS-4224, along with a saturating concentration, 10 µg/mL, of an anti-PD-L1 antibody (durvalizumab, Medimmune/AstraZeneca) in a separate test. Following exposure, samples were either left unstimulated (resting) or stimulated (activated) for 24 hours at 37°C, 5.0% CO_2 with 500 ng IFN-γ, activating monocytes to increase express of PD-L1 receptors. For flow analysis, samples were incubated with Human TruStain Fc Receptor (FcX) solution (Biolegend, San Diego, CA) for 5 minutes at room temperature and then stained with CD45 (HI30) APC-eFlour 780 (Thermo Fisher Scientific, Waltham, MA), CD14 (M5E2) APC (Biolegend, San Diego, CA), CD3 (SK7) PerCP-Cy5.5 (Biolegend, San Diego, CA), CD4 (RPA-T4) BV421 (Biolegend, San Diego, CA), CD8 (RPA-T8) FITC (Biolegend, San Diego, CA), Fixable Viability Dye eFluor 506 (Thermo Fisher Scientific, Waltham, MA), and custom PD-L1 (atezolizumab, Genentech) PE (Southern Biotech, Birmingham, AL) for 30 minutes at room temperature in a final volume of 200 µL per well. Samples were then lysed and fixed with 1.5mL of 1X prepared BD LyseFix buffer (Becton Dickinson, Franklin Lakes, NJ) and finally washed with BD Pharmingen Stain Buffer (FBS) (Becton Dickinson, Franklin Lakes, NJ) and acquired on an LSRFortessa X-20 (Becton Dickinson, Franklin Lakes, NJ).

Tumor cell lysis assay

GFP-MDA-MB-231 cells were obtained from Essen Biosciences and maintained in DMEM with 4.5 g/L glucose, L-glutamine and sodium pyruvate (Corning, Manassas, VA) supplemented with non-essential amino acids, 10 units/mL penicillin, 10 µg/mL streptomycin, and 10% heat-inactivated FBS from HyClone (Logan, UT). The cells were seeded in PrimeSurface 3D culture 96-well ultra-low attachment (ULA) round bottom plates (sBio, Hudson, NH) three days before the initiation of the co-culture tumor lysis assay at a concentration of 10^4 cells/well using complete AIM V media (ThermoFisher Scientific, Waltham, MA) supplemented with 10% HI-FBS. Following a three-day incubation period at 37°C, 5% CO_2, titrations of PD-L1 antibody (atezolizumab, Genentech) and GS-4224 were dispensed in triplicate within the pre-seeded 96-well plates. Next, $CD8^+$ T cells (STEMCELL Technologies, Vancouver, Canada) at a density of 2 x 10^6 cells/mL were added to the plates for a final assay volume of 200 µL (0.1% DMSO normalization) and an effector to target (E:T) ratio of 10 to 1. The co-culture plates were loaded into the IncuCyte S3 (Essen BioScience Inc., Ann Arbor, MI) housed within a 37°C, 5% CO_2 incubator and using both phase contrast and green fluorescence image channels at a 10X objective, reads were taken every 4 hours for 5 to 6 days. Tumor lysis was normalized to dimethyl sulfoxide (0 % lysis) and to 10 µg/mL of PD-L1 antibody (100 % lysis).

In vivo mouse tumor studies

Efficacy

Human PD-L1-overexpressing murine MC38 (HuMC38) colorectal tumor cells (Crown Bioscience) were implanted subcutaneously into the right flank of female C57BL/6 mice. Once tumors reached a mean volume of ≈50 mm^3, mice were randomized (12 mice per group) and treatments were administered as an intraperitoneal injection. Tumor volumes were measured twice weekly in two dimensions using a caliper until end of study, and the volume was expressed in mm^3 using the formula: V = (L x W x W) / 2, where V is tumor volume, L is tumor length (the longest tumor dimension) and W is tumor width (the longest tumor dimension perpendicular to L).

Data was analyzed with SPSS (Statistical Product and Service Solutions) version 18.0 (IBM, Armonk, NY, U.S.) and were two-sided. P-values < 0.05 were considered to be statistically significant. The GS-4224 group was compared to the vehicle control group. The atezolizumab group was compared to the isotype control group.

Assessment of target occupancy on tumor

Tumors were collected from mice and washed in PBS with extra tissues removed (i.e. blood vessel, fat and fascia). In each well of a sterile 6-well plate (Corning), the tumor was placed in dissociation media (Murine Tumor Dissociation Kit, Miltenyi). Tumors were sliced until small tumor pieces of ~ 1 mm^3 were obtained and transferred to gentleMACS C tubes (Miltenyi) and placed on ice until digestion. Once all the tumors had been sliced, C tubes were transferred to gentleMACS Octo Dissociator with Heaters (Miltenyi). Dissociation program (37_c_m_TDK_1) was selected for tumor digestion. Samples were re-suspended and added to a cell strainer (Corning) placed above a 50 mL centrifuge tube (Corning). Cells were washed through the cell strainer with 10 mL of wash buffer (10% FBS, Gibco; 40 mM EDTA, Boston BioProducts; PBS without calcium and magnesium, GE Healthcare) to obtain single cell suspensions.

One million tumor cells were re-suspended in 15 mL centrifuge tubes (Corning) with 200 µL of Staining Buffer (BD Biosciences) and 1 µg/mL Mouse Fc Block (Purified rat α-mouse CD16/CD32, BD Biosciences). Tubes were incubated for 15 mins in the dark at 4°C. For flow analysis, cells were then stained with CD45 (30-F11) BUV661 (BD Biosciences), CD3 (145-2C11) BUV395 (BD Biosciences), CD11b (M1/70) BV605 (Biolegend), human PD-L1 (MIH1) PE-Cy7 (Thermo Fisher Scientific) and Fixable Viability Dye eF-506 (Thermo Fisher Scientific) for additional 30 mins at 4°C. After washing step, cells were resuspended with 150 µL of Staining Buffer and data was acquired on LSRFortessa X-20 (BD Biosciences). Target occupancy (TO) is calculated as the loss of PD-L1 fluorescence signal compared to control tumor cells.