SUPPLEMENTARY MATERIALS

Table S1. Differential expression analysis for CD8 T cells.

Table S2. Differential expression analysis for Treg CD4 T cells.

Table S3. Differential expression analysis for non-Treg CD4 T cells.

Table S4. Differential expression analysis for NK cells.

Table S5. List of Genes Used to Generate NK Cell Activation Scores.

SUPPLEMENTARY MATERIALS AND METHODS:

Detailed co-culture generation and culture: High grade serous ovarian cancer (HGSC) tumor samples were obtained directly from the BWH frozen section room, and research samples were taken immediately after the frozen section was completed to minimize cold ischemic time. Tumors were mechanically dissociated, first with a scalpel blade to 2-4mm sections, and then by crushing with the butt of a 10mL syringe. The cell suspension was then diluted in DMEM (Life Tech # 11965-092), 10% FBS (Sigma # F2442-500), 1% Penicillin Streptomycin (Life Tech # 15140-122), and 2.5mg/mL Type II Collagenase (Life Technologies # 17101015). This homogenate was then shaken on a horizontal platform for 20 minutes at 37°C. The homogenate was filtered through a 100um filter (Corning # 352360) and spun for three minutes at 1500RPM. The media was aspirated, and the cell pellet was incubated for five minutes at room temperature in 1X Red Blood Cell Lysis buffer (Biolegend #420301), and spun again for three minutes at 1500RPM. The lysis buffer was aspirated, and if sufficient red blood cell lysis had occurred, the cells were resuspended in five times the pellet volume of DMEM, 10% FBS, and 1% Pen/Strep. The cell suspension underwent manual counting after an aliquot was diluted with Trypan blue with efforts to count tumor spheres and single cells. A portion of the suspension was then spun at 1500RPM, and snap frozen in FBS/10%DMSO for later flow cytometry analysis. The remaining portion of the suspension with the appropriate cell number was spun at 1500RPM for three minutes, the media was aspirated and the pellet was diluted to a concentration of $6x10^6$ cells (or organoids)/mL in DMEM, 10% FBS, 1% Pen/Strep, and 30ng/mL of IL-2 (Peprotech #200-02) mixed with 15% Matrigel (Corning #356231). This cell suspension was then plated into 48 well plates (USA Scientific # CC7672-7548) with enough wells for each treatment and assay. 40uL of suspension was added per well. These plates were placed in a 37°C incubator to allow for settling of the sample into the plate. During that time, appropriate volumes of media containing drugs to be tested were prepared. All drug

preparations were prepared in DMEM, 10% FBS, 1% Pen/Strep, and 30ng/mL of IL-2. Drugs included Anti-PD-1 (Selleck #A2005), IgGEn Isotype Control (provided by Eli Lilly), anti-PD-L1 (LY3300054), bispecific anti-PD-1/PD-L1 (LY3434172), and BAY-299 (MedChemExpress # HY-107424). All antibodies were used at a final concentration of 10μg/mL. Combination experiments for antibodies were performed by adding 20μg/mL IgGEN (IgG+IgG) or 10μg/mL each of anti-PD-1 and anti-PD-L1. Combination experiments of small molecules and antibodies were performed by combining 10μg/mL of each antibody with either 1μM BAY-299 or an equivalent volume of DMSO as to what was added with the BAY-299 as a vehicle control. Once the cell suspensions had been allowed to settle, the drug containing media was gently added over the suspensions. Brightfield images were taken, and the co-cultures were then incubated for 96 hours at 37°C. After 96 hours, media and cultures were harvested for ELISA or flow cytometry analysis, respectively. At 94 hours, half of the media in each well to be used for flow cytometry analysis was aspirated, snap frozen, and stored at -80°C. Protein transporter inhibitor (Fisher #00-4980-03) was added to the remaining media at a concentration of 1:500, and the cells were incubated with this inhibitor for 2 hours prior to being harvested for flow cytometry analysis. Flow cytometry harvest is described below. For cell sorting or ELISA, the protein transporter step was omitted.

Single-cell RNA-seq data analysis: The single-cell RNA sequencing reads were aligned to human GRCh38 reference genome and quantified using Cell Ranger (v3.1.0) from 10X Genomics. Cell filtering and clustering were performed with the Seurat (1) package (v3.0.0). Cells with less than 200 genes detected or with larger than 10% mitochondrial genes detected were excluded. After preprocessing, the sequencing yielded an expression matrix of 3,828 cells by 21,644 genes for the parent tumor. Hashtag demultiplexing was performed using the HTODemux function in Seurat with a positive quantile of 0.997, yielding an expression matrix of 8,199 cells by 20,198 genes. Specifically, 2,447 cells were indented in the IgG group, 1,853

cells in the anti-PD-L1 group, 2,145 cells in the anti-PD-1 group, and 1,754 cells in the Bispecific group. Gene expression was normalized by total count number and multiplied by 10,000 as a scaling factor. The top 2,000 most variable genes were selected for downstream dimension reduction and clustering. Dimension reduction and visualization were performed with the Uniform Manifold Approximation and Projection (UMAP) with 30 principle components as input. Unsupervised cell clustering was performed using the shared nearest neighbor clustering algorithm with a resolution of 0.8. Differentially expressed genes were identified with the Wilcoxon Rank Sum test and are used as marker genes (Tables S1-S4). Known cell type marker genes, including *CD3E* for T cells, *CD8A* for CD8+ T cells, *CD4* for CD4+ T cells, *FOXP3* for Tregs, *MKI67* for Ki67+ T cells, *ADGRE1* for Macrophages, *KLRK1* for NK cells, and *CD19* and *CD79A* for B cells were utilized for cell type annotation. At a fine-grain resolution, T cells were separately clustered and annotated for cell status, including *HAVCR2* (TIM3), *LAG3* and *PDCD1* (PD-1) for exhausted CD8+ T cells, *TCF7* and *IL7R* for naïve/memory T cells, and *TCF7* and *PDCD1* (PD-1) for progenitor exhausted T cells. To reveal underlying pathways, GO enrichment analysis was performed using the differential genes with clusterProfiler (2) (v3.10.1). KEGG pathway or biological process enrichments were performed with the hypergeometric model. Multiple hypothesis was corrected by using the Benjamini-Hochberg Procedure. Pseudotime trajectories were constructed by diffusion maps using R package destiny (3). Marker genes in the naïve and progenitor exhausted CD8 groups were used as the input gene list for constructing diffusion maps. The first and second components from the diffusion map were used for visualization of CD8+ T cell differentiation. The correlation between reference gene (*GZMB* and *IFNG* for activation, *HAVCR2* and *PDCD1* for exhaustion) and all variable genes across all CD8+ T cells was computed using the compute.network function in bigScale2 (4) with scaled gene expression as input. The CD8+ T cell activation/exhaustion signature consisted of the top 50 genes with the highest correlation coefficient with reference genes. The

NK cell activation signatures consisted of 22 known NK cell activation genes (Table S5) (5). AddModuleScore function in Seurat was used for scoring of individual cells.

IFNγ ELISA: IFNγ ELISAs were performed using Biolegend's ELISA MAX Deluxe Set Human IFNγ kit (# 430104) following the manufacturer protocol. Undiluted media supernatants were used and loaded in triplicate. The standard was prepared for a range of 7.8-1000pg/mL. Nunc MaxiSorp plates (Biolegend # 423501) were used along with Biolegend wash (# 421601) and stop buffers (# 423001). Statistical analysis was performed in GraphPad Prism using paired t-tests.

Preparation and staining of co-cultures for flow cytometry analysis: After 96 hours of treatment and the two-hour pulse with protein transporter inhibitor described above, the co-cultures were harvested into appropriately labeled tubes according to treatment. A vial of the frozen parent tumor was thawed and divided into three tubes for staining. Cultures and parent tumor were spun at 1500 RPM for 3 minutes, the media was aspirated, and the cultures were washed with 500uL of Biolegend's Cell Staining Buffer (Cat. #420201). Cultures were spun again at 1500RPM for 3 minutes, the wash buffer was aspirated, and 100uL of BD Brilliant stain buffer (Cat # 563794) with Zombie Aqua Live Dead dye (Biolegend Cat #423101) were added to the co-cultures. An unstained control and individual tubes with co-culture for single stains and fluorescence minus one (FMO) controls for all intracellular antibodies were also prepared and appropriately carried forward. These were incubated for 20 minutes at room temperature in the dark. During the 20-minute incubation, cocktails of surface marker antibodies for Panels 1, 2, and 3 (shown in the table below) were prepared. At the 20-minute timepoint, the appropriate surface antibody mixture or single antibody was added to each tube of co-culture, the mixture was vortexed, and these tubes were incubated at room temperature for 20 minutes. After 20

minutes, one mL of cell staining buffer was added to each tube to wash the cells, the tubes were spun at 1500 RPM for 3 minutes, and supernatant was aspirated. 100uL of Biolegend's Fluorofix Buffer (Cat. #422101) were added to each tube, and the tubes were incubated at room temperature for 20 minutes in the dark. During this incubation, the intracellular antibody cocktails for Panels 1, 2, and 3 (shown in the table below) were prepared in Biolegend's 1X Intracellular Staining Permeabilization Wash Buffer (Cat. #421002). At the 20-minute timepoint, one mL of Permeabilization Wash Buffer was added to each tube to wash the cells, the tubes were spun at 1500 RPM for 3 minutes, and supernatant was aspirated. The appropriate intracellular antibody mixes or single antibody was added to each tube, the cells were vortexed, and the tubes were incubated for 20 minutes at room temperature in the dark. At twenty minutes, one mL of Permeabilization Wash Buffer was added to each tube, the tubes were spun at 1500 RPM for 3 minutes, and supernatant was aspirated. 400 uL of Cell Staining buffer were added to each tube, and the pellets were mixed and then pipetted into flow cytometry tubes (Falcon #352054). Prior to analysis, as an extra option for compensation, tubes with compensation beads (Fisher #01-2222-41) stained with each fluorophore were also prepared. All tubes were analyzed on a BD LSR Fortessa Flow Cytometer in the DFCI Flow Cytometry Core Facility with uniform voltages for all samples in each panel for each tumor. The unstained sample, single stain controls, bead controls, and FMOs were analyzed first to check gates and later for compensation. Each control was run individually with each panel to allow for proper compensation controls at the voltage used for each panel. For each panel, each sample in the panel was run at the same voltage and the same number of cells were analyzed. At a minimum 30,000 cells were analyzed, but in most cases between 50,000 and 100,000 cells per sample were uniformly run for each panel. Flow data was analyzed using FlowJo software. Compensation matrices were set up for each tumor for each panel using either the single stain controls or beads from that particular voltage depending on the quality of the single stain

controls. Gating was based off of FMOs and single stain controls. Statistical analysis of results was performed in GraphPad Prism using paired t-tests to compare treatments.

Human Antibody Panels

Human Panel 1			
Target	**Clone**	**Fluorophore**	**Catalog Number**
Surface Markers			
Live Dead Dye	--	Zombie Aqua	Biolegend 423101
CD279 (PD-1)	EH12.2H7	Brilliant Violet 421	Biolegend 329919
CD274 (PD-L1)	29E.2A3	Brilliant Violet 785	Biolegend 329735
CD366 (TIM3)	7D3	Alexa Fluor 647	BD 565559
CD69	FN50	Brilliant Violet 711	Biolegend 310943
CD3	OKT3	Brilliant Violet 605	Biolegend 317321
CD4	PRA-T4	Brilliant Violet 650	Biolegend 300535
CD8	RPA-T8	APC/Cy7	Biolegend 301015
CD56	5.1H11	PerCP/Cyanine 5.5	Biolegend 362505
CD45	2D1	FITC	Biolegend 368507
EPCAM	9C4	PE	Biolegend 324205
Intracellular Markers			
IFN-γ	B27	PE/Cy7	Biolegend 506517
Ki67	Ki-67	PE/Dazzle	Biolegend 350533

Human Panel 2			
Target	**Clone**	**Fluorophore**	**Catalog Number**
Surface Markers			
Live/Dead Dye		Zombie Aqua	Biolegend 423101
CD279 (PD-1)	EH12.2H7	Brilliant Violet 421	Biolegend 329919
CD274 (PD-L1)	29E.2A3	Brilliant Violet 785	Biolegend 329735
CD366 (TIM3)	7D3	Alexa Fluor 647	BD # 565559
CD14	W6D3	Brilliant Violet 711	Biolegend 301837
CD33	P67.6	Brilliant Violet 605	Biolegend 366611
CD15	MC-480	Brilliant Violet 650	Biolegend 323033
CD19	HIB19	APC/Cy7	Biolegend 302217
CD11c	3.9	PerCP/Cyanine 5.5	Biolegend 301623
CD45	2D1	FITC	Biolegend 368507
EPCAM	9C4	PE	Biolegend 324205
Intracellular Markers			
IFN-γ	B27	PE/Cy7	Biolegend 506517
Ki67		PE/Dazzle	Biolegend 350533

Human Panel 3			
Target	**Clone**	**Fluorophore**	**Catalog Number**
Surface Markers			
Live Dead Dye	--	Zombie Aqua	Biolegend 423101
CD69	FN50	Brilliant Violet 711	Biolegend 310943
CD3	OKT3	Brilliant Violet 605	Biolegend 317321
CD4	PRA-T4	Brilliant Violet 650	Biolegend 300535
CD8	RPA-T8	APC/Cy7	Biolegend 301015
CD56	5.1H11	PerCP/Cyanine 5.5	Biolegend 362505
CD45	2D1	FITC	Biolegend 368507
EPCAM	9C4	PE	Biolegend 324205
CD107A	H4A3	Brilliant Violet 421	Biolegend 328625
Intracellular Markers			
IFN-γ	B27	PE/Cy7	Biolegend 506517
Ki67	Ki-67	PE/Dazzle	Biolegend 350533
Granzyme B	QA16A02	APC	Biolegend 372203

Preparation and staining of co-cultures and parent tumor for flow cytometry sorting: For the parent tumor, tumor cells were thawed and stained as follows. For co-cultures, 96 hours after initial treatment and without any protein transporter inhibitor treatment, cultures were scraped from the plate into appropriate tubes. Cultures and parent tumor were spun at 1500 RPM for 3 min. For the cultures, the media was aspirated and frozen at -80°C for later ELISA as a backup. For the parent tumor, the media was discarded. All cells were then washed with 500uL of Biolegend's Cell Staining Buffer. Cultures were spun again at 1500RPM for 3 minutes, the wash buffer was aspirated, and 100uL of Cell Staining buffer with Zombie Aqua Live dead dye were added to the cells. An unstained control and individual tubes with co-culture for single stain were also prepared and appropriately carried forward. These were incubated for 10 minutes on ice in the dark. During the 20-minute incubation, cocktails of surface markers were prepared.

For the single cell RNA sequencing experiments, the staining was as follows. At the 20-minute timepoint, for the organoid co-cultures FITC anti-CD45 antibody was added to each tube

of co-culture, the mixture was vortexed, and these tubes were incubated at room temperature for 20 minutes. For the parent tumor, a mixture of FITC anti-CD45, PE anti-EPCAM, BV711 anti-CD14, and BV605 anti-CD33 was added or single antibody controls were added, the mixture was vortexed, and these tubes were incubated at room temperature for 20 minutes. After 20 minutes, one mL of cell staining buffer was added to each tube to wash the cells, the tubes were spun at 1500 RPM for 3 minutes, and supernatant was aspirated. 400 uL of cell staining buffer were added to each tube, the cells were filtered through a filter top into a flow cytometry tube, and these cells were immediately transported to the DFCI flow cytometry core. There, viable CD45+ cells were sorted on the BD FACS ARIA II cell sorter using the 100-μm nozzle at 20 psi into 100uL of 0.4% BSA in PBS for both the parent tumor and organoid cultures. In addition, for the parent tumor, tumor and stromal cells were sorted into a separate tube.

For the bulk RNA sequencing experiment on samples from patient 20-35, the staining was as follows. Parent tumor was thawed and organoid co-cultures were harvested at the 96 hour timepoint, and both were washed in cell staining buffer. Then an antibody mixture in cell staining buffer was added containing Zombie Aqua, FITC anti-CD45, PE anti-EPCAM, PerCPCy5.5 anti-CD56, BV605 anti-CD3, BV650 anti-CD4, APC-Cy7 anti-CD8, and BV 421 anti-CD25 (Biolegend Cat. #302629). The mixture was vortexed, and these tubes were incubated at room temperature for 20 minutes. Single antibody controls were also prepared. After 20 minutes, one mL of cell staining buffer was added to each tube to wash the cells, the tubes were spun at 1500 RPM for 3 minutes, and supernatant was aspirated. 400 uL of cell staining buffer were added to each tube, the cells were filtered through a filter top into a flow cytometry tube, and these cells were immediately transported to the DFCI flow cytometry core. There, multiple cell sets were harvested including viable CD8+ T cells, viable CD4+CD25+ T cells, viable CD4+CD25- T cells, viable NK cells, and viable tumor cells. Cells were sorted directly into fresh buffer RLT with β-mercaptoethanol from the Qiagen RNeasy Mini kit (Cat.

#74104) and stored at -80°C until all cell types for each treatment were sorted. RNA was prepared following the Qiagen RNeasy Mini kit protocol with on-column DNAse digest and bulk RNA sequencing was performed in duplicate for each sample at Novogene using their low sample concentration protocol.

Parent Tumor RNA Preparation: Snap frozen parent tumor samples were stored at -80°C until needed. Prior to RNA preparation, each parent tumor was pulverized using a Covaris tissue pulverizer. Fresh buffer RLT from the Qiagen RNeasy Mini kit (Cat. #74104) containing β-mercaptoethanol was added to each sample. RNA was prepared following the Qiagen RNeasy Mini kit protocol with on-column DNAse digest and bulk RNA sequencing was performed in duplicate for each sample at Genewiz using their standard protocol.

RNA-seq data analysis: The RNA sequencing reads were aligned to the human GRCh38 reference genome using STAR (6) (v2.6.1). Genes were quantified using RSEM (7) (v1.3.3). Gene differential expression analysis was performed using DESeq2 (8) (v1.22.2) with integer expected read count matrix as input. Gene Set Enrichment Analysis was performed using log2FoldChange-ranked gene list with clusterProfiler. For the parent tumors, the IFNγ signature score was quantified as $\frac{\sum log(TPMg+1)}{6}$ where TPMg is the expression level of *IFNG, IDO, CXCL10, CXCL9, HLA-DR1*, and *STAT1* in transcripts per million (TPM) (9).

Cell and organoid line culture: All cell lines were originally obtained from ATCC, except for KHYG1 which was obtained through MTA from Dr. Carolyn Hurly**.** Organoids were generated previously (10). All cell lines and organoids tested negative for mycoplasma by PCR in August 2020. All experiments on these lines occurred in the first twenty passages post-thawing. The

KHYG1 NK cell line (11) was grown in flasks in RPMI with glutamine (Gibco Cat. No. 11875119), 10% FBS (Sigma Cat. No. F2442-500), 1% pen/strep (Gibco Cat. No. 15140-122), and 20ng/mL IL-2 (Peprotech Cat. No. 200-02). NK92 cells were grown in PRIME-XV NK Cell CDM (Fujifilm Cat. No. 91215) supplemented with 1% pen/strep and 20ng/mL IL-2 in flasks. Jurkat cells were grown in RPMI, 10% FBS, and 1% Pen/Strep. OVCAR8, SKOV3, CaOV3, and CaOV4 cells were grown in RPMI, 10%FBS, and 1% pen/strep. Organoid lines 17-116 and 17-121 were grown as described previously (10).

ATAC-seq and data analysis: KHYG1 cells were expanded and treated with either vehicle (DMSO) or 0.1uM BAY-299 for 96 hours. 50,000 cells from the vehicle and treatment groups were then viably frozen in FBS containing 10% DMSO. ATAC-seq was performed as described previously by the Center for Functional Cancer Epigenetics at Dana-Farber (12). The ATAC sequencing reads were aligned to human GRCh38 genome using bwa mem (13) (v0.7.15). To extract accessible regions across the genome, we called ATAC-seq peaks using MACS2 (14) (v2.2.7.1). Peaks in different samples were merged as a union peak set using bedtools merge (14). Read count matrix was extracted for each peak from the bam file of each sample using bedtools multicov. Differential peaks were called using DESeq2 with a p-value < 0.05 as we treated each peak as a gene. Transcription factors (TFs) binding to differentially accessible regions were extracted using the Cistrome DB Toolkit (15). The overlap score was calculated as a MinMax scaling Giggle score for TFs. Gene enrichment analysis for differential ATAC-seq peaks were performed using Cistrome-GO (16).

siRNA treatment: KHYG1 cells were plated in regular media on day 0 for siRNA treatment to test BRD1 antibodies for western. Control siRNA (Qiagen AllStars Negative control Cat. #1027280) or one of two different *BRD1* specific siRNAs (Dharmacon Cat. #D-006963-02-0002 and D-006963-04-0002) were mixed in Opti-Mem (Gibco Cat. #31985070) and Lipofectamine

RNAiMax (Invitrogen Cat. #13778100) and added to the KHYG1 cells on days 0 and 1. The cells were allowed to incubate for an additional 48 hours after the last transfection and then harvested for western blot analysis.

Western Blot: KHYG1 cells treated with siRNA as above or with vehicle or 0.1uM BAY-299 for 96 hours were lysed in NETN300 lysis buffer (300mM NaCL, 50mM Tris pH 8.0, 1mM EDTA, and 0.05% NP40). The lysates were spun at 15,000 RPM for 15 minutes, and the soluble fractions were saved and concentrations normalized using Laemmli buffer (Boston Bioproducts Cat. #BP-110NR) for western blot. Equal amounts of each sample were loaded into 4-12% Bis-Tris gels (Invitrogen Cat. #NP0336BOX) and run using MOPS running buffer (Invitrogen Cat. #NP0001). Gels were transferred to 0.45um nitrocellulose membranes, and the membranes were stained with Bethyl's Rabbit anti-BRD1 antibody (Cat. #A302-366) and Sigma's Tubulin Antibody (Cat. #T-5168).

Cell and organoid line survival analysis: Cell lines and organoid lines were grown in regular media as described above and plated into 96 well plates as monolayers for cell lines or in 20% Matrigel as a slurry for organoids. On day 0, one set of untreated cells was treated with Cell Titer Glo (Promega Cat. #G7572) and the luminescence was read on a ClarioStar plate reader as a baseline measurement for later growth rate correction. The other wells were treated in triplicate with 0, 0.005, 0.05, 0.1, 0.5, 1, or 5uM BAY-299 for 96 hours. At 96 hours, Cell Titer Glo was added to the plates and the luminescence was read on a ClarioStar plate reader. For each organoid or cell line, these treatments were repeated twice in triplicate for each dose. The growth rate corrected GR50 area over the curve (AOC)(17) was calculated and plotted in GraphPad prism.

KHYG1 cell and organoid line co-culture experiments: KHYG1 cells were grown in regular culture media containing either vehicle or 0.5uM BAY-299 for 48 hours. Co-cultures were generated with a 1:1 ratio of NK: tumor cells. At the 48 hour timepoint, 5,000-10,000 vehicle or drug treated KHYG1 cells were plated per well of a round bottom 96 well plate (Corning Cat. No. 0720095) either alone or together with either 5,000-10,000 OVCAR8 cells or approximately 5,000-10,000 cells from 17-116 organoid cultures. The single cells or co-cultures were grown in 100uL of KHYG1 media containing either vehicle or 0.5uM BAY-299 per well depending on the previous treatment of the KHYG1 cells for another 48 hours. At the 48 hour timepoint, the media was harvested for ELISA and the cells harvested for flow cytometry according to the above co-culture protocols. No protein transporter inhibitor treatment was used prior to harvest. ELISA was performed as described above. Cells were stained for flow cytometry using FITC anti-CD45, PerCP-Cy5.5 anti-CD56, PE anti-EPCAM, BV 421 anti-CD107A, PE Cy7 anti-IFNγ, PE Dazzle anti-Ki67, and APC anti-GZMB as above and Zombie NIR viability dye (Biolegend Cat. No. 423105) and analyzed as described above.

KHYG1 and OVCAR8 co-culture cell death experiments: KHYG1 cells were treated with vehicle or 0.5uM BAY-299 for 108 hours with media being refreshed at 48 hours. At the 108 hour timepoint, KHYG1 cells were counted, and 5000 KHYG1 cells were plated with 5000 OVCAR8 cells in 100uL of KHYG1 cell media containing the appropriate vehicle or drug based on the previous treatment per well of a 96 well plate. The cells were incubated together at 37°C for 6 hours, harvested and washed in cell staining buffer, stained with PerCPCy5.5 anti-CD56, PE anti-EPCAM, Zombie NIR, and Apotracker Green dye (Biolegend Cat. No. 427401), washed, and immediately analyzed on a BD LSR Fortessa Flow Cytometer for Apotracker positive dead cells.

***In Vivo* Max Tolerated Dose (MTD) Study**: DFCI Institutional Care and Use Committee (IACUC) protocol 19-016 was used to determine the maximum tolerated dose of BAY-299. Sixteen 7-9 week old FVB-N mice were obtained from Taconic (Germantown, NY) and sorted into four groups for the initial study. Four groups with four mice each were treated with either vehicle (10% NMP (Fisher Cat # 390682500), 90%PEG400 (Sigma Cat # 202398-500G)), 10 mg/kg, 30 mg/kg, 90 mg/kg, or later 150mg/kg BAY-299 (MedChemExpress HY-107424) for 11-15 days using once daily oral gavage for dosing. Animals were monitored by weight daily for the duration of the treatment and carefully observed for adverse effects. At the end of the study, mice were euthanized according to the IACUC approved protocol. For the vehicle treated group, the spleens were harvested, crushed with the butt of a syringe, pelleted, and stained as described for solid tumors in the flow cytometry analysis section below. This allowed for testing of all antibodies for efficacy and appropriate concentration for flow analysis prior to the efficacy study.

Flow cytometry analysis of STOSE mice ascites, spleen, and solid tumor immune composition: Mice from vehicle and BAY-299 treatment groups were euthanized according to the IACUC approved protocol. Ascites samples from both groups were aspirated, pelleted, and incubated with red blood cell lysis buffer (Biolegend Cat. # 420301) at room temperature for 5 minutes. The cells were pelleted, washed once in media, pelleted, washed once in cell staining buffer (Biolegend Cat. #420201), and then stained as described below.

For solid tumors, the tumors were mechanically dissociated, first with a scalpel blade to 2-4mm sections, and then by crushing with the butt of a 10mL syringe. The cell suspensions were then diluted in DMEM (Life Tech # 11965-092), 10% FBS (Sigma # F2442-500), 1% Penicillin Streptomycin (Life Tech # 15140-122), and 2.5mg/mL Type II Collagenase (Life

Technologies # 17101015). This homogenate was then shaken on a horizontal platform for 20 minutes at 37°C. The homogenate was filtered through a 100um filter (Corning # 352360) and spun for three minutes at 1500RPM. The media was aspirated, and the cell pellet was incubated for five minutes at room temperature in 1X Red Blood Cell Lysis buffer, and spun again for three minutes at 1500RPM. The lysis buffer was aspirated, and if sufficient red blood cell lysis had occurred, the cells were washed once in cell staining buffer and stained as described below.

For spleens, the spleens were mechanically dissociated, first with a scalpel blade to 2-4mm sections, and then by crushing with the butt of a 10mL syringe. The spleen cell suspensions were then filtered through a 100um filter and spun for three minutes at 1500RPM. The media was aspirated, and the cell pellet was incubated for five minutes at room temperature in 1X Red Blood Cell Lysis buffer, and spun again for three minutes at 1500RPM. The lysis buffer was aspirated, and if sufficient red blood cell lysis had occurred, the cells were washed once in cell staining buffer and stained as described below.

Cocktails of surface marker antibodies for Panels 1 and 3 (shown in the table below) were prepared in advance. Cell suspensions were spun again at 1500RPM for 3 minutes, the cell staining buffer was aspirated, and 100uL of BD Brilliant stain buffer (Cat. # 563794) with viability dye and the surface antibody cocktail (see tables below) were added to the ascites, spleen, or solid tumor cell suspensions. The cell-antibody mixtures were vortexed, and these tubes were incubated at room temperature for 20 minutes. After 20 minutes, one mL of cell staining buffer was added to each tube to wash the cells, the tubes were spun at 1500 RPM for 3 minutes, and supernatant was aspirated. 100uL of Biolegend's Fluorofix Buffer (Cat. #422101) were added to each tube, and the tubes were incubated at room temperature for 20 minutes in the dark. During this incubation, the intracellular antibody cocktails for Panels 1 and 3 (shown in the table below) were prepared in Biolegend's 1X Intracellular Staining Permeabilization Wash Buffer (Cat. #421002). At the 20-minute timepoint, one mL of Permeabilization Wash Buffer was added to each tube to wash the cells, the tubes were spun at 1500 RPM for 3 minutes, and

supernatant was aspirated. The appropriate intracellular antibody mixes or single antibody was added to each tube, the cells were vortexed, and the tubes were incubated for 20 minutes at room temperature in the dark. At twenty minutes, one mL of Permeabilization Wash Buffer was added to each tube, the tubes were spun at 1500 RPM for 3 minutes, and supernatant was aspirated. 400 uL of Cell Staining buffer were added to each tube, and the pellets were mixed and then pipetted into flow cytometry tubes (Falcon #352054). Prior to analysis, tubes with compensation beads (Fisher #01-2222-41) stained with each fluorophore were also prepared. All tubes were analyzed on a BD LSR Fortessa Flow Cytometer in the DFCI Flow Cytometry Core Facility with uniform voltages for all samples in each panel for each tumor. Each control was run individually with each panel to allow for proper compensation controls at the voltage used for each panel. For each panel, each sample in the panel was run at the same voltage and the same number of cells were analyzed. At a minimum 30,000 cells were analyzed, but in most cases between 50,000 and 400,000 cells per sample were uniformly run for each panel depending on tissue type. Flow data was analyzed using FlowJo software. Compensation matrices were set up for each tumor for each panel using the beads from that particular voltage. Gating was based off of bead controls. Statistical analysis of results was performed in GraphPad Prism using unpaired t-tests to compare treatments.

Murine Antibody Panels

Murine Panel 1			
Target	**Clone**	**Fluorophore**	**Catalog Number**
Surface Markers			
Live Dead Dye	--	Zombie Aqua	Biolegend 423101
CD279 (PD-1)	29F.1A12	Brilliant Violet 421	Biolegend 135217
CD274 (PD-L1)	B7-H1	Brilliant Violet 785	Biolegend 124331
CD366 (TIM3)	RMT3-23	Alexa Fluor 647	Biolegend 119705
NKp46	H1.2F3	Brilliant Violet 711	Biolegend 137621
CD3	17A2	Brilliant Violet 605	Biolegend 100237
CD4	RM4-5	Brilliant Violet 650	Biolegend 100545
CD8	53-6.7	APC/Cy7	Biolegend 100713
NK-1.1	PK136	PerCP/Cyanine 5.5	Biolegend 108727
CD45	30-F11	FITC	Biolegend 103107

EPCAM	G8.8	PE	Biolegend 118205
Intracellular Markers			
IFNγ	XMG1.2	PE/Cy7	Biolegend 505825
Ki67	16A8	PE/Dazzle	Biolegend 652427

Murine Panel 3			
Target	**Clone**	**Fluorophore**	**Catalog Number**
Surface Markers			
Live Dead Dye	--	Zombie Aqua	Biolegend 423101
NKp46	H1.2F3	Brilliant Violet 711	Biolegend 137621
CD3	17A2	Brilliant Violet 605	Biolegend 100237
CD4	RM4-5	Brilliant Violet 650	Biolegend 100545
CD8	53-6.7	APC/Cy7	Biolegend 100713
NK-1.1	PK136	PerCP/Cyanine 5.5	Biolegend 108727
CD45	30-F11	FITC	Biolegend 103107
EPCAM	G8.8	PE	Biolegend 118205
CD107A	1D4B	Brilliant Violet 421	Biolegend 121617
Intracellular Markers			
IFNγ	XMG1.2	PE/Cy7	Biolegend 505825
Ki67	16A8	PE/Dazzle	Biolegend 652427
Granzyme B	QA16A02	APC	Biolegend 372203

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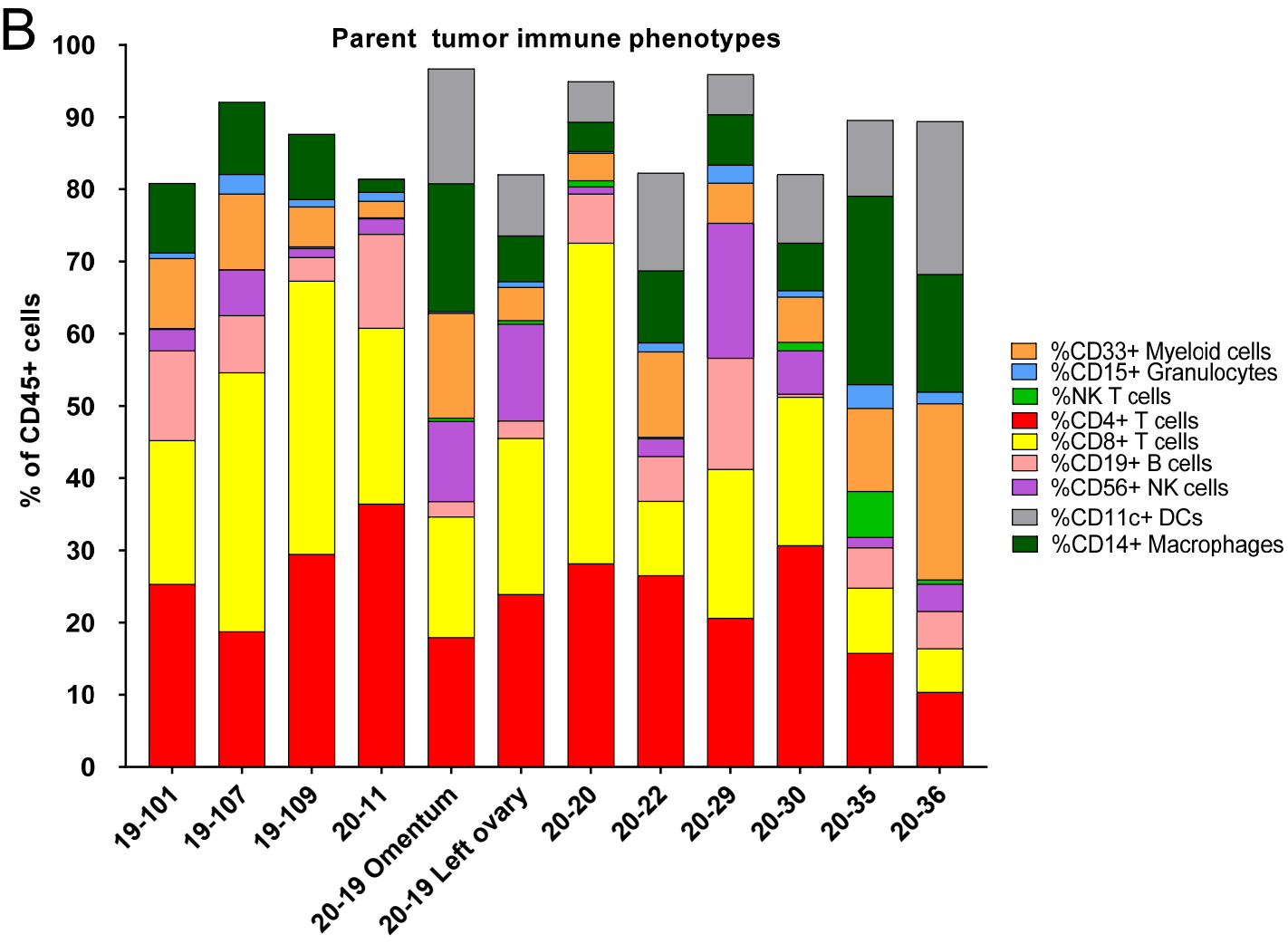
Figure S1

A

Patient	Tumor Site	Tumor Type
19-100	Omental metastasis	Untreated HGSC
19-101	Omental metastasis	Untreated HGSC
19-107	Peritoneal biopsy	Untreated HGSC
19-109	Omental metastasis	Untreated HGSC
20-11	Omental metastasis	Untreated HGSC
20-19	Left ovary	Untreated HGSC
20-19	Omental metastasis	Untreated HGSC
20-20	Left ovary	Carcinosarcoma s/p neoadjuvant chemotherapy, left ovary listed as HGSC only
20-22	Left ovary	HGSC s/p neoadjuvant chemotherapy
20-29	Left ovary	Untreated HGSC
20-30	Right ovary	Untreated HGSC
20-35	Right ovary	Untreated HGSC
20-36	Omental metastasis	Untreated HGSC

B



C

Figure S1

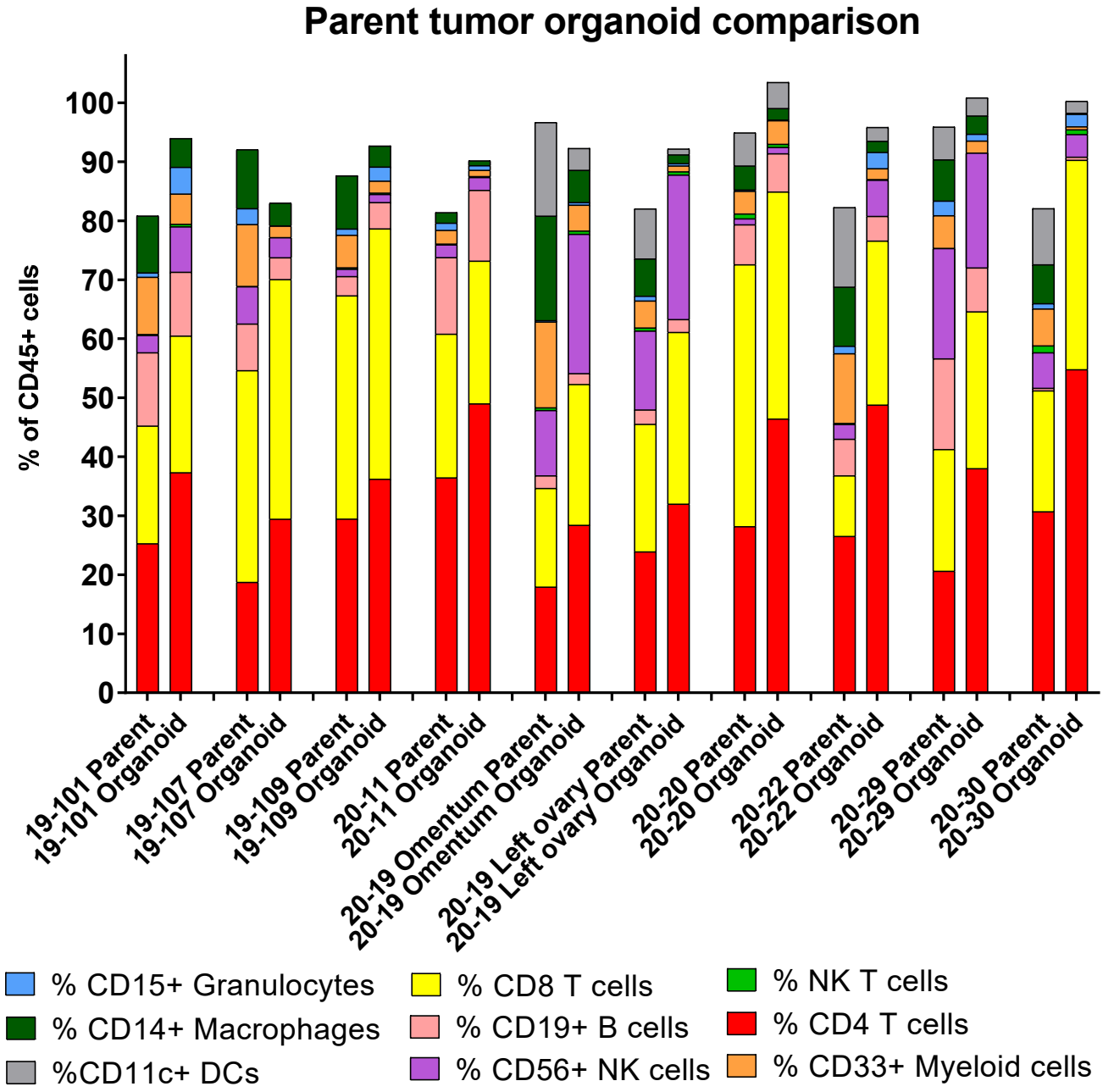

Parent tumor organoid comparison
% of CD45+ cells
0
10
20
30
40
50
60
70
80
90
100
19-101 Parent
19-101 Organoid
19-107 Parent
19-107 Organoid
19-109 Parent
19-109 Organoid
20-11 Parent
20-11 Organoid
20-19 Omentum Parent
20-19 Omentum Organoid
20-19 Left ovary Parent
20-19 Left ovary Organoid
20-20 Parent
20-20 Organoid
20-22 Parent
20-22 Organoid
20-29 Parent
20-29 Organoid
20-30 Parent
20-30 Organoid
% CD15+ Granulocytes
% CD14+ Macrophages
%CD11c+ DCs
% CD8 T cells
% CD19+ B cells
% CD56+ NK cells
% NK T cells
% CD4 T cells
% CD33+ Myeloid cells

Figure S1. Properties of cases analyzed: A) Solid tumors were obtained from 12 high grade serous ovarian cancer (HGSC) patients. The de-identified code is in the left column, the tumor site is in the middle column, and the treatment status and histologic diagnosis is in the right column. **B)** Immune profiles of all parent tumors. Parent tumor immune composition was analyzed by flow cytometry. The percentage of each immune cell type present as a percent of CD45+ cells for each parent tumor is shown here. The color code for immune cells is shown on the right. **C)** Flow cytometry analysis of parent tumors (left) and control treated organoid co-cultures (right) for all immune cells. Parent tumor and control treated immune composition was analyzed by flow cytometry. The percentage of each immune cell type present as a percent of CD45+ cells for each parent tumor and organoid co-culture is shown here. The color code for immune cells is shown below the bar graph. Tumors 19-101, 19-107, 19-109, and 20-11 did not have dendritic cell (DC) analysis in their panels. The organoid cultures for 20-35 and 20-36 did not have a myeloid panel and are not shown in Figure S1C, but the parent tumors of these cases were shown in Figure S1B.

Figure S2

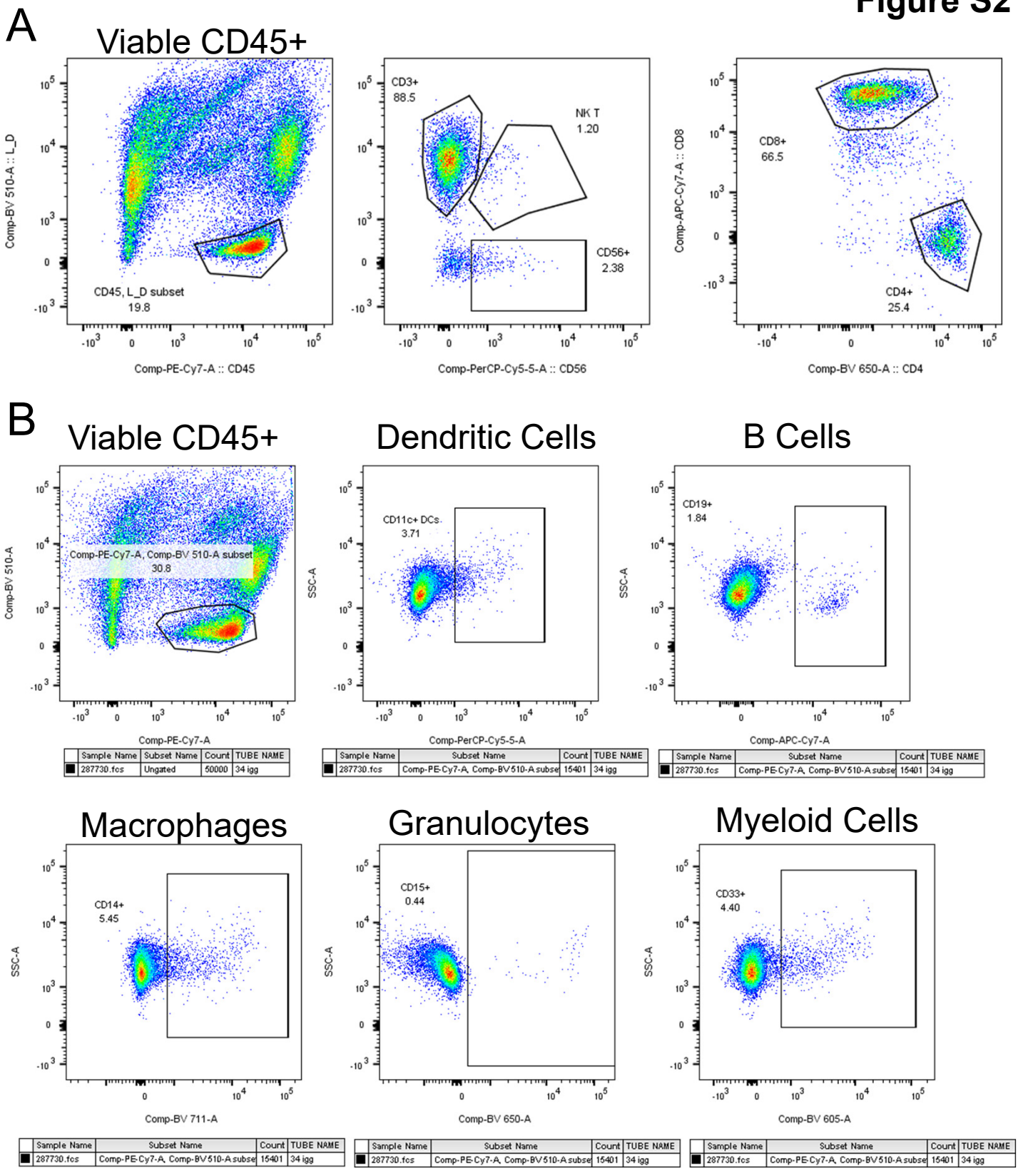

A
Viable CD45+
Comp-BV 510-A :: L_D
Comp-PE-Cy7-A :: CD45
CD45, L_D subset
19.8
CD3+
88.5
NK T
1.20
CD56+
2.38
Comp-PerCP-Cy5-5-A :: CD56
Comp-APC-Cy7-A :: CD8
CD8+
66.5
CD4+
25.4
Comp-BV 650-A :: CD4
B
Viable CD45+
Dendritic Cells
B Cells
Comp-BV 510-A
Comp-PE-Cy7-A, Comp-BV 510-A subset
30.8
Comp-PE-Cy7-A
SSC-A
CD11c+ DCs
3.71
Comp-PerCP-Cy5-5-A
CD19+
1.84
Comp-APC-Cy7-A
Sample Name | Subset Name | Count | TUBE NAME
287730.fcs | Ungated | 50000 | 34 igg
287730.fcs | Comp-PE-Cy7-A, Comp-BV 510-A subse | 15401 | 34 igg
287730.fcs | Comp-PE-Cy7-A, Comp-BV 510-A subse | 15401 | 34 igg
Macrophages
Granulocytes
Myeloid Cells
CD14+
5.45
Comp-BV 711-A
CD15+
0.44
Comp-BV 650-A
CD33+
4.40
Comp-BV 605-A
287730.fcs | Comp-PE-Cy7-A, Comp-BV 510-A subse | 15401 | 34 igg
287730.fcs | Comp-PE-Cy7-A, Comp-BV 510-A subse | 15401 | 34 igg
287730.fcs | Comp-PE-Cy7-A, Comp-BV 510-A subse | 15401 | 34 igg

Figure S2. Flow cytometry gating strategy: A) For T, NK, and NK T analysis, a representative gating strategy from 20-19 left ovary is shown. First viable CD45+ cells were gated. From those, CD3+ CD56- T cells, CD3+ CD56+ NK T cells, and CD56+ CD3- NK cells were gated. From the CD3+ CD56- T cells, CD4+ and CD8+ T cells were gated. Intracellular stains and immuno-oncologic receptors were gated from these populations. **(B)** For CD33+ myeloid cells, CD15+ granulocytes, CD19+ B cells, CD11c+ dendritic cells, and CD14+ macrophages a representative gating strategy from 20-19 omentum is shown. First viable CD45+ cells were gated. From those, the above respective populations were gated as shown.

Figure S3

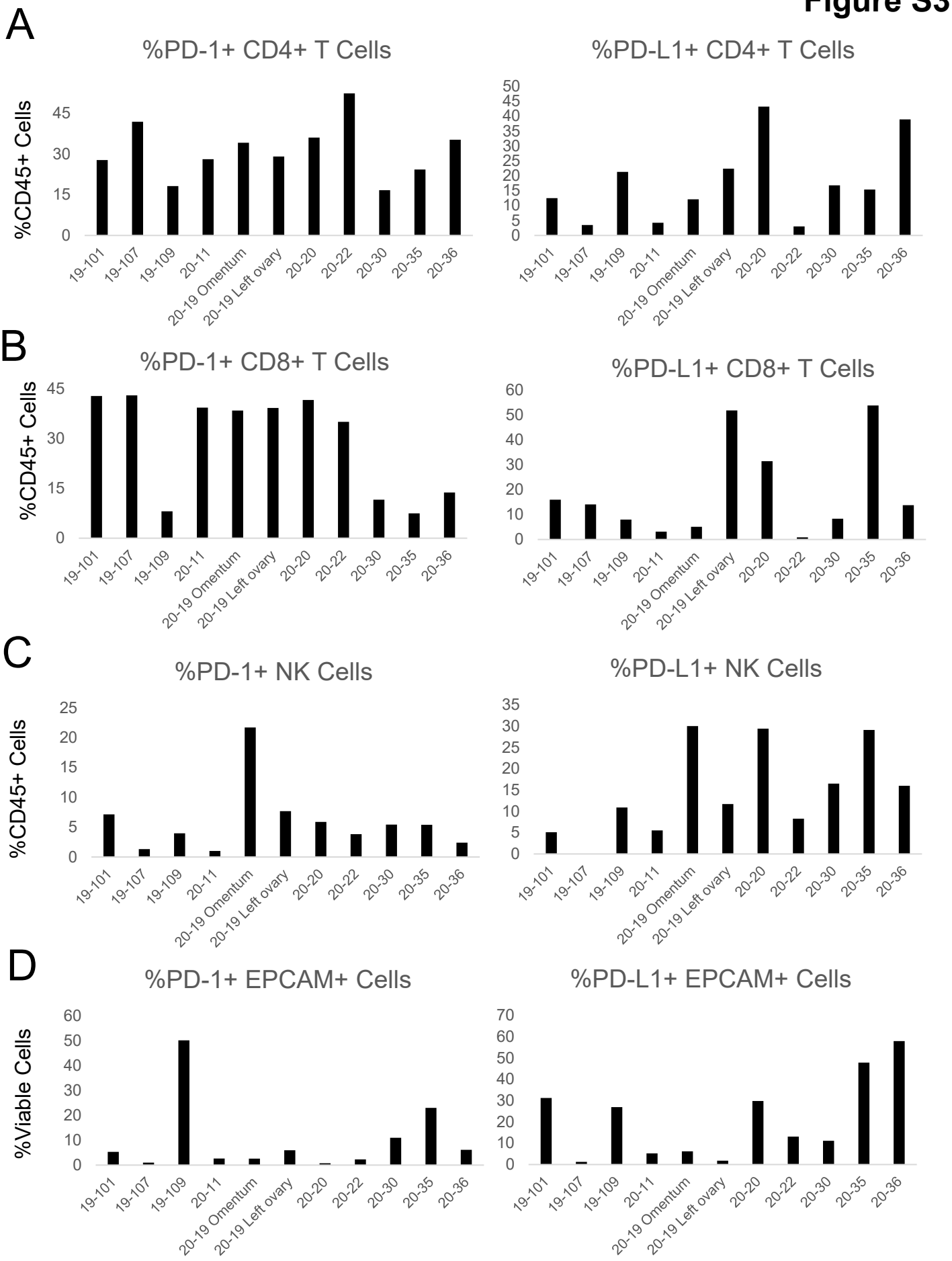

A
%PD-1+ CD4+ T Cells
%CD45+ Cells
0
15
30
45
19-101
19-107
19-109
20-11
20-19 Omentum
20-19 Left ovary
20-20
20-22
20-30
20-35
20-36
%PD-L1+ CD4+ T Cells
0
5
10
15
20
25
30
35
40
45
50
B
%PD-1+ CD8+ T Cells
%CD45+ Cells
0
15
30
45
%PD-L1+ CD8+ T Cells
0
10
20
30
40
50
60
C
%PD-1+ NK Cells
%CD45+ Cells
0
5
10
15
20
25
%PD-L1+ NK Cells
0
5
10
15
20
25
30
35
D
%PD-1+ EPCAM+ Cells
%Viable Cells
0
10
20
30
40
50
60
%PD-L1+ EPCAM+ Cells
0
10
20
30
40
50
60
70

Figure S3. Drug target receptor status on T and NK cells in organoid co-cultures: The PD-1 and PD-L1 expression level was assessed by flow cytometry and gated from the populations defined in Figure S2 and is shown for control treated organoids for **(A)** CD4, **(B)** CD8, **(C)** CD56+ NK cells as a percentage of viable CD45+ cells, and **(D)** EPCAM+ tumor cells.

Figure S4

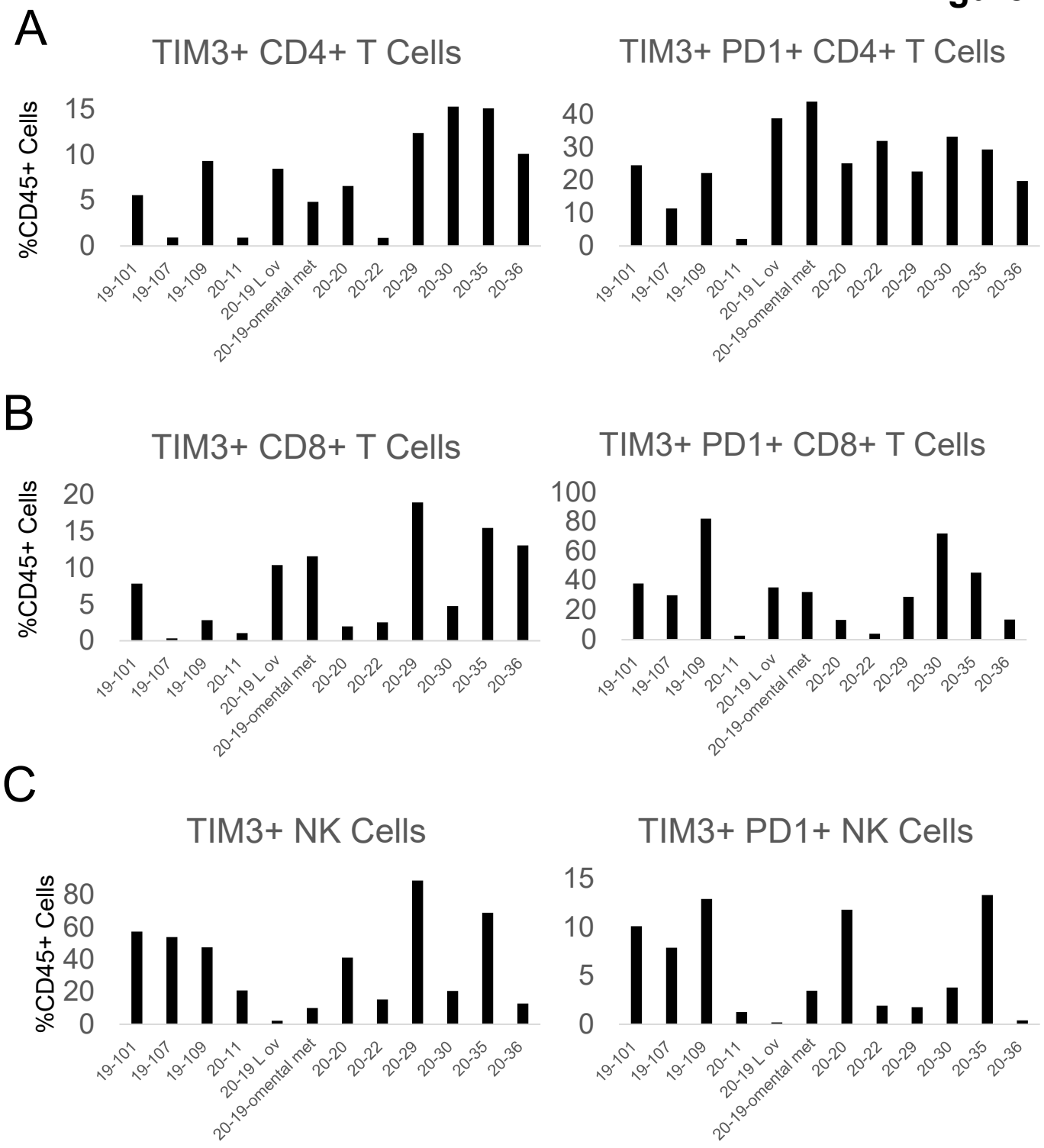

A
TIM3+ CD4+ T Cells
%CD45+ Cells
15
10
5
0
19-101
19-107
19-109
20-11
20-19 L ov
20-19-omental met
20-20
20-22
20-29
20-30
20-35
20-36
TIM3+ PD1+ CD4+ T Cells
40
30
20
10
0
B
TIM3+ CD8+ T Cells
20
15
10
5
0
TIM3+ PD1+ CD8+ T Cells
100
80
60
40
20
0
C
TIM3+ NK Cells
80
60
40
20
0
TIM3+ PD1+ NK Cells
15
10
5
0

Figure S4. Exhaustion marker receptor status on T and NK cells in organoid co-cultures: The TIM3 and TIM3/PD-1 co-expression level was assessed by flow cytometry and gated from the populations defined in Figure S2 and is shown for control treated organoids for **(A)** CD4 T cells, **(B)** CD8 T cells, and **(C)** CD56+ NK cells as a percentage of viable CD45+ cells.

Figure S5

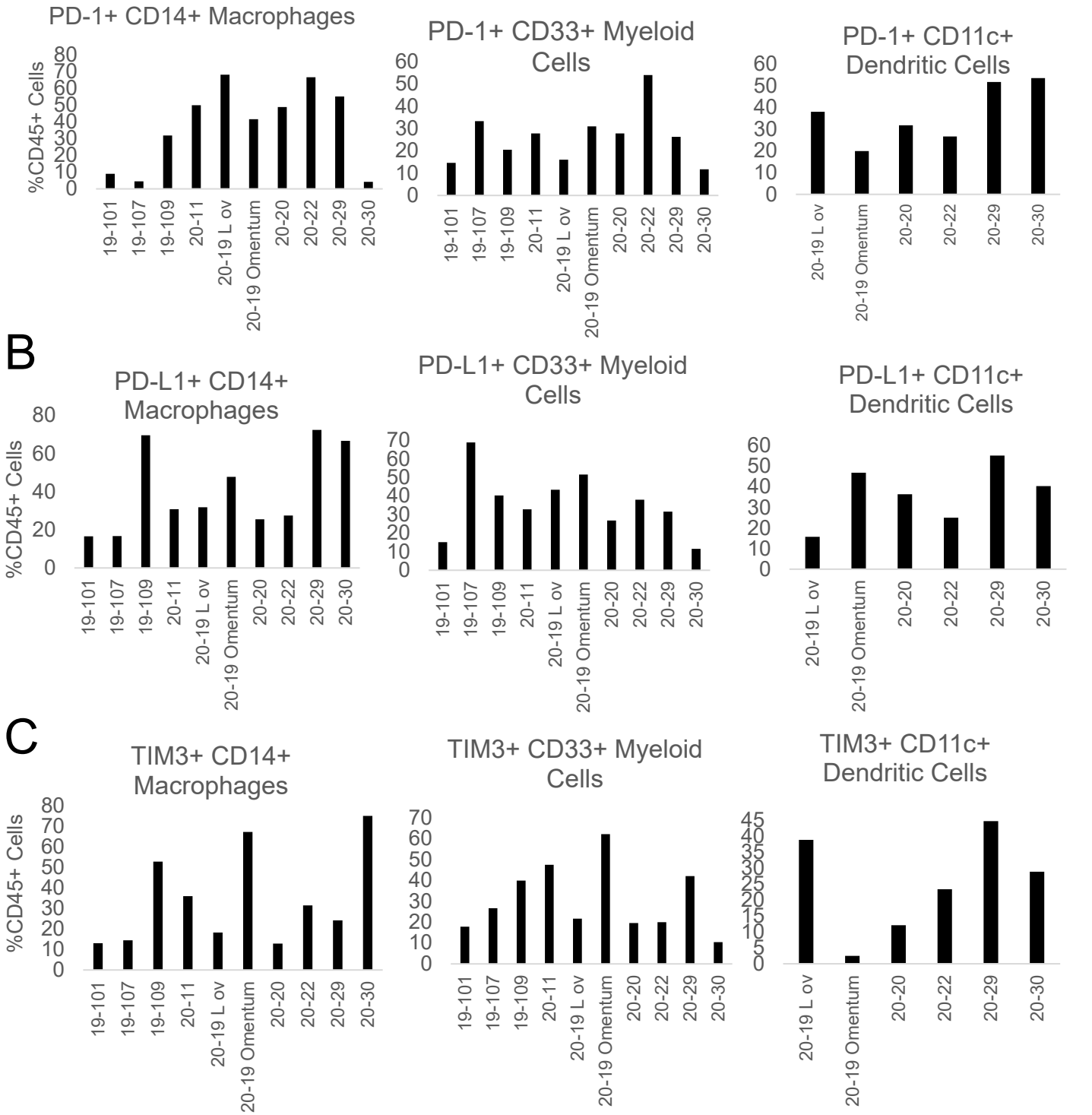
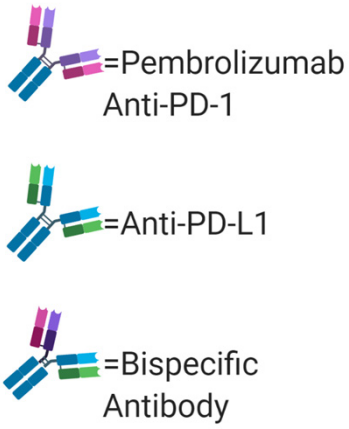

A
PD-1+ CD14+ Macrophages
%CD45+ Cells
PD-1+ CD33+ Myeloid Cells
PD-1+ CD11c+ Dendritic Cells
B
PD-L1+ CD14+ Macrophages
PD-L1+ CD33+ Myeloid Cells
PD-L1+ CD11c+ Dendritic Cells
C
TIM3+ CD14+ Macrophages
TIM3+ CD33+ Myeloid Cells
TIM3+ CD11c+ Dendritic Cells
19-101
19-107
19-109
20-11
20-19 L ov
20-19 Omentum
20-20
20-22
20-29
20-30

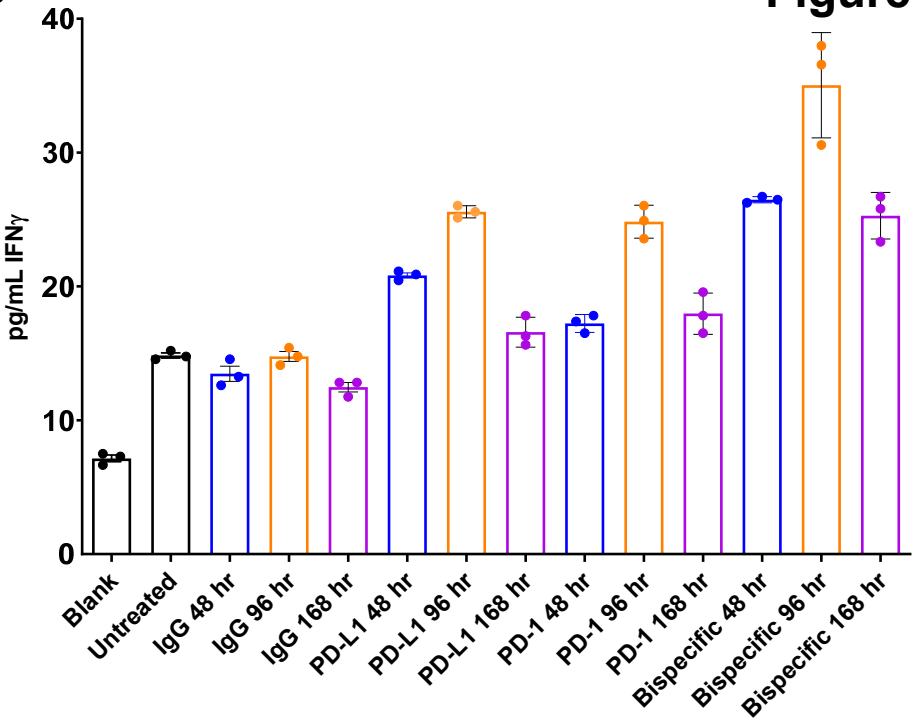
Figure S5. Immune checkpoint blockade receptor expression on myeloid cells, macrophages, and dendritic cells: Immune checkpoint blockade receptor level expression was assessed by flow cytometry and gated from the populations defined in Figure S2 and is shown for control treated organoids for CD14, CD33, and CD11c positive cells for **(A)** PD-1, **(B)** PD-L1, and **(C)** TIM3 as a percentage of viable CD45+ cells.

Figure S6

A



B



C

Comparison	Paired T test p-value
IgGvs PD-1	0.0092**
IgG vs PD-L1	0.0027**
IgG vs Bispecific	0.0008***
IgG vs PD-1+PD-L1	0.0443*
IgG vs IgG+IgG	0.7038 NS
Bispecific vs PD-1	0.0002***
Bispecific vs PD-L1	0.0015**
Bispecific vs PD-1+PD-L1	0.0464*
Bispecific vs IgG+IgG	0.026*
PD-1 vs PD-1+PD-L1	0.8885 NS
PD-L1 vs PD-1+PD-L1	0.3563 NS
IgG+IgG vs PD-1+PD-L1	0.0584 NS

D

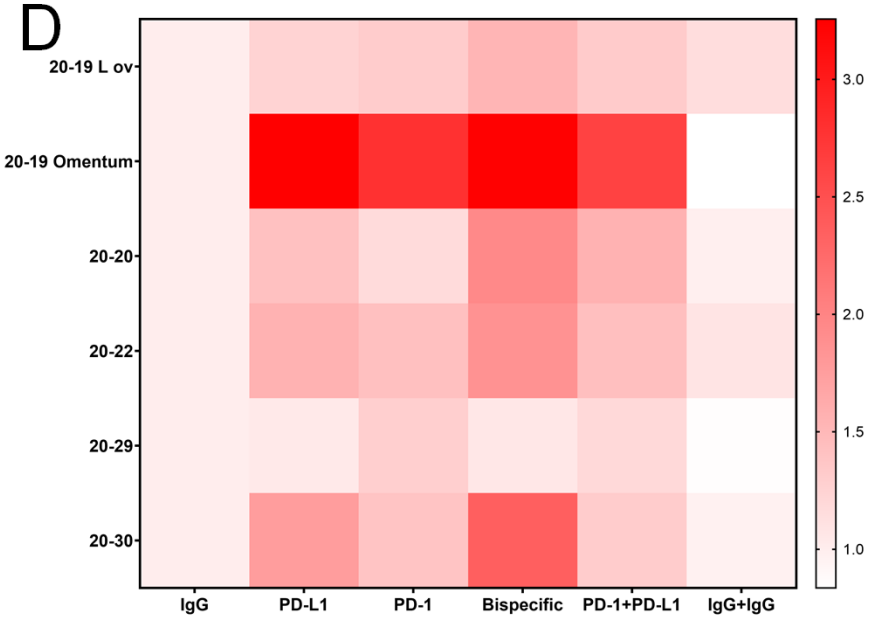
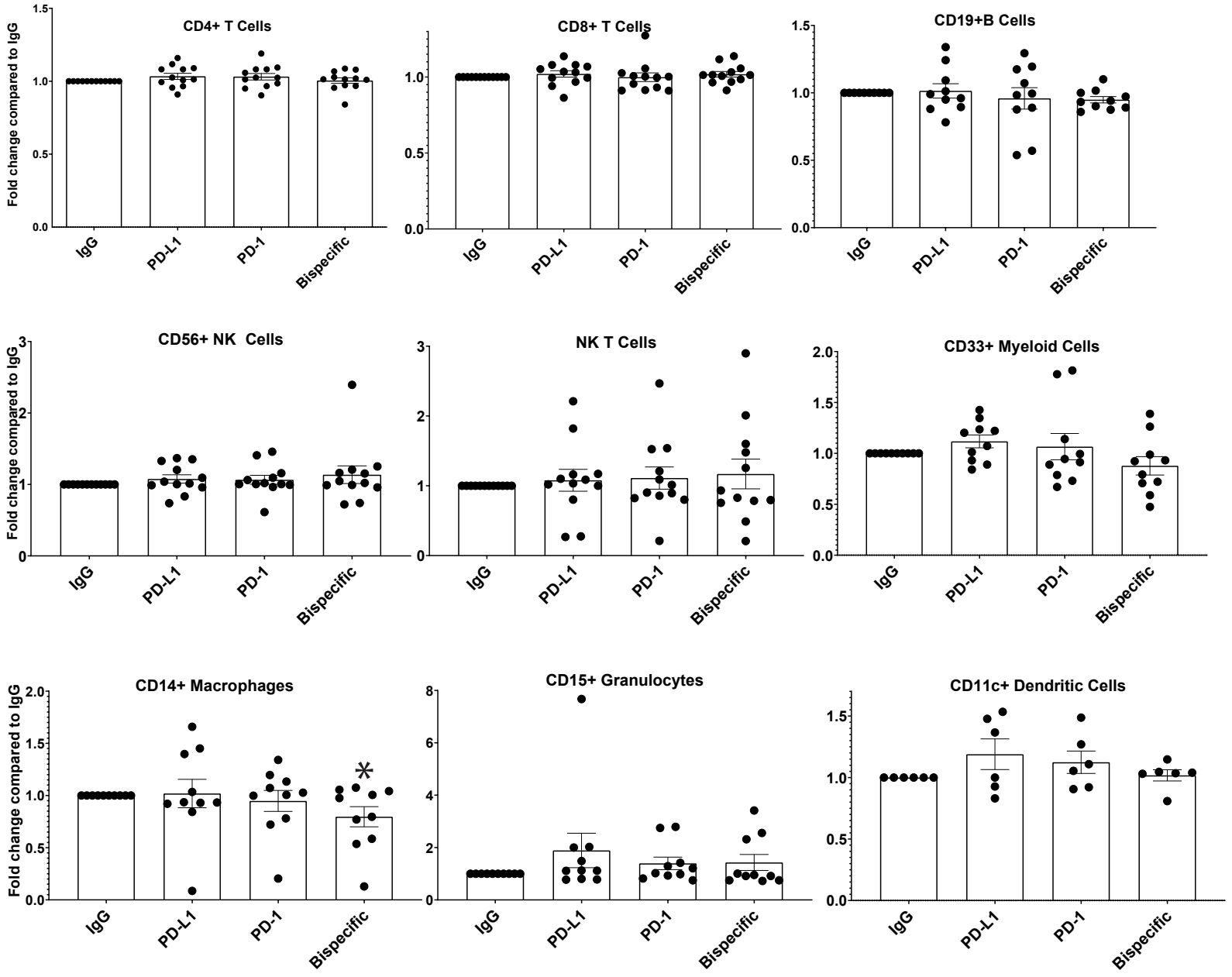
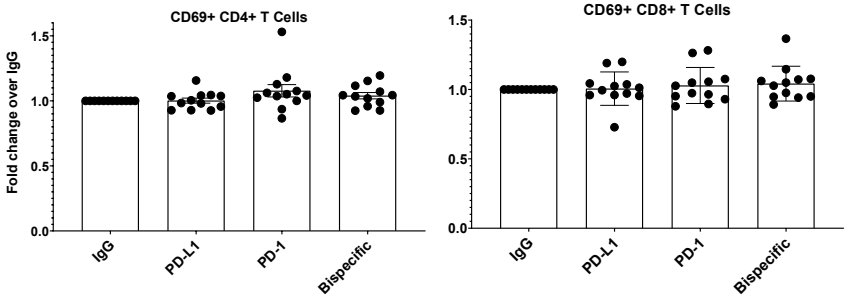


Figure S6. Antibody map, ELISA optimization, and ELISA heatmap for double antibody control treatments: A) Map of antibodies used in this study. **B)** To choose the optimal timepoint for analysis, IFNγ ELISAs were performed on treated organoid co-cultures at 48, 96, and 168 hours post treatment. The co-culture media supernatants underwent IFNγ ELISA analysis shown here as the average pg/mL of IFNγ for the treatment with error bars representing standard error between three replicates. This ELISA is from tumor 20-11. **C)** p-values were generated for the ELISA comparisons between treatments shown in Figure 2A using a paired t-test. The p-values for all comparisons are shown in the table here. **D)** IFNγ ELISA analysis was performed on media from organoid co-cultures treated with IgG control, anti-PD-1, anti-PD-L1, bispecific, anti-PD-1+anti-PD-L1, and IgG+IgG antibodies. IFNγ amounts are shown here as a heatmap for each experiment where all six treatments were performed normalized to the IgG control.

A


CD4+ T Cells
CD8+ T Cells
CD19+B Cells
CD56+ NK Cells
NK T Cells
CD33+ Myeloid Cells
CD14+ Macrophages
CD15+ Granulocytes
CD11c+ Dendritic Cells
Fold change compared to IgG
IgG
PD-L1
PD-1
Bispecific
*

B


CD69+ CD4+ T Cells
CD69+ CD8+ T Cells
Fold change over IgG
IgG
PD-L1
PD-1
Bispecific

C

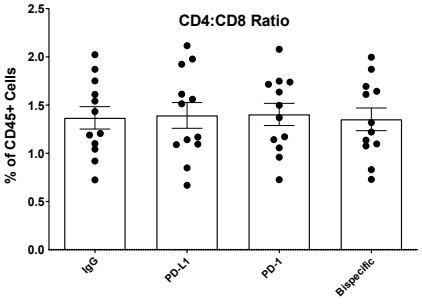

CD4:CD8 Ratio
% of CD45+ Cells
IgG
PD-L1
PD-1
Bispecific

Figure S7. Ratio of cell types across treatment: A) Percentages of all immune cells were assessed from the viable CD45 positive cells for all organoid co-cultures after treatment, and the fold change of each treatment compared to the IgG control is shown here. Error bars represent standard error of the mean. All differences were insignificant except the CD14+ Macrophage difference between IgG and bispecific antibody. *$p<0.05$ calculated by a paired t-test. **B)** Percentages of CD4 and CD8 T cells expressing CD69 were assessed by flow cytometry from the viable CD45 positive cells for all organoid co-cultures after treatment, and the fold change of each treatment compared to the IgG control is shown here. Error bars represent standard error of the mean. **C)** Percentages of CD4 and CD8 cells were assessed from the viable CD45 positive cells for all organoid co-cultures after treatment, and the ratio of CD4 to CD8 cells was calculated and is shown here normalized to the IgG control. Error bars represent standard error of the mean.

Figure S8

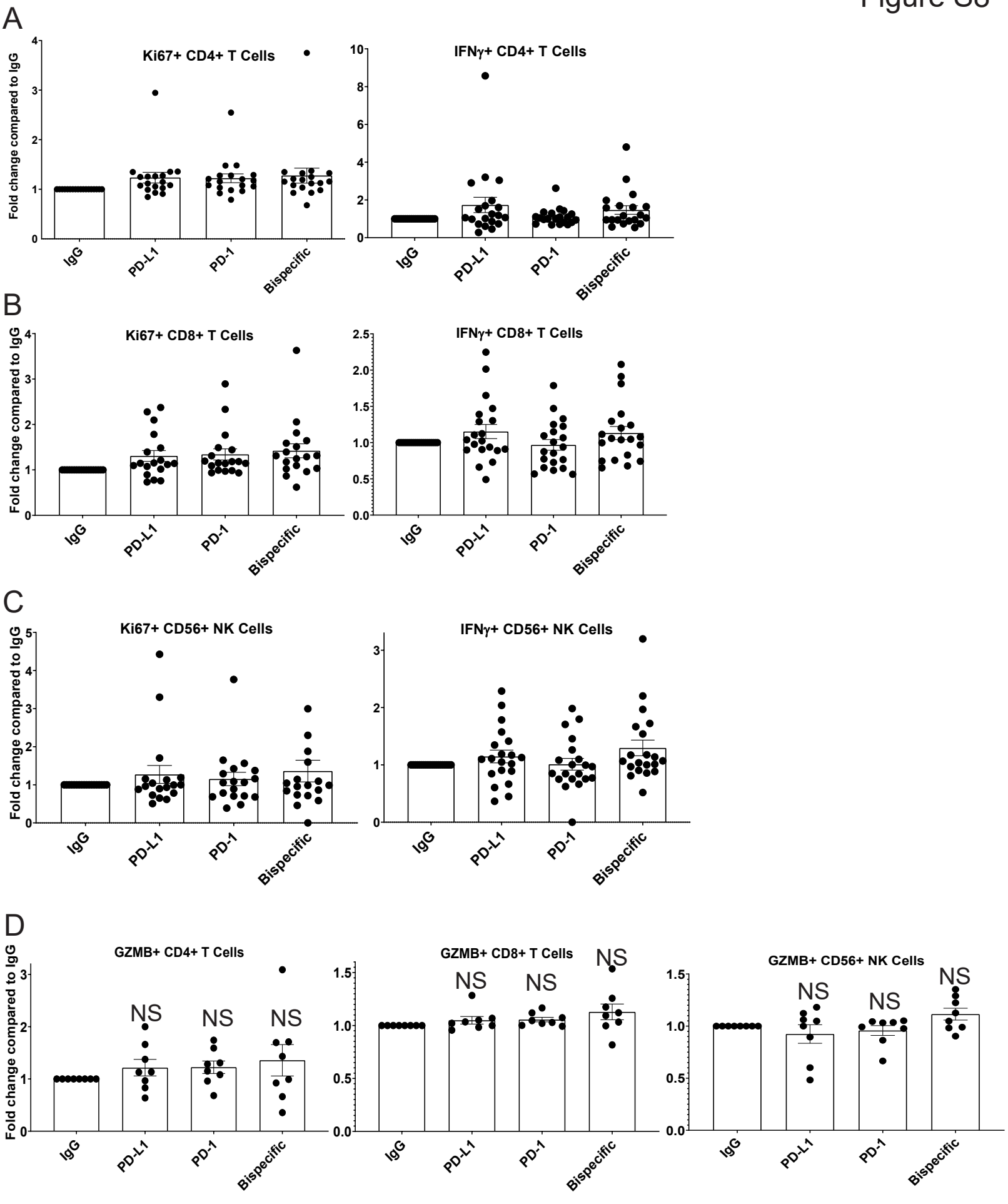

A
Ki67+ CD4+ T Cells
IFNγ+ CD4+ T Cells
Fold change compared to IgG
B
Ki67+ CD8+ T Cells
IFNγ+ CD8+ T Cells
C
Ki67+ CD56+ NK Cells
IFNγ+ CD56+ NK Cells
D
GZMB+ CD4+ T Cells
GZMB+ CD8+ T Cells
GZMB+ CD56+ NK Cells
NS
IgG
PD-L1
PD-1
Bispecific

Figure S8. Granzyme B, Ki67, and IFNγ expression in T and NK cells post-immune checkpoint blockade treatment: A) Flow cytometry analysis in CD4 T cells as a percent of CD45+ cells for all treatments normalized to the IgG control for Ki67 expression (left) and IFNγ expression (right). **B)** Flow cytometry analysis in CD8 T cells for all treatments as a percent of CD45+ cells normalized to the IgG control for Ki67 expression (left) and IFNγ expression (right). **C)** Flow cytometry analysis in CD56 positive NK cells for all treatments as a percent of CD45+ cells normalized to the IgG control for Ki67 expression (left) and IFNγ expression (right). **D)** Flow cytometry analysis as a percent of CD45+ cells for granzyme B (GZMB) for all treatments normalized to the IgG control in CD4 T cells, CD8 T cells, and CD56 positive NK cells. NS=Not significant. Error bars represent standard error of the mean.

Figure S9

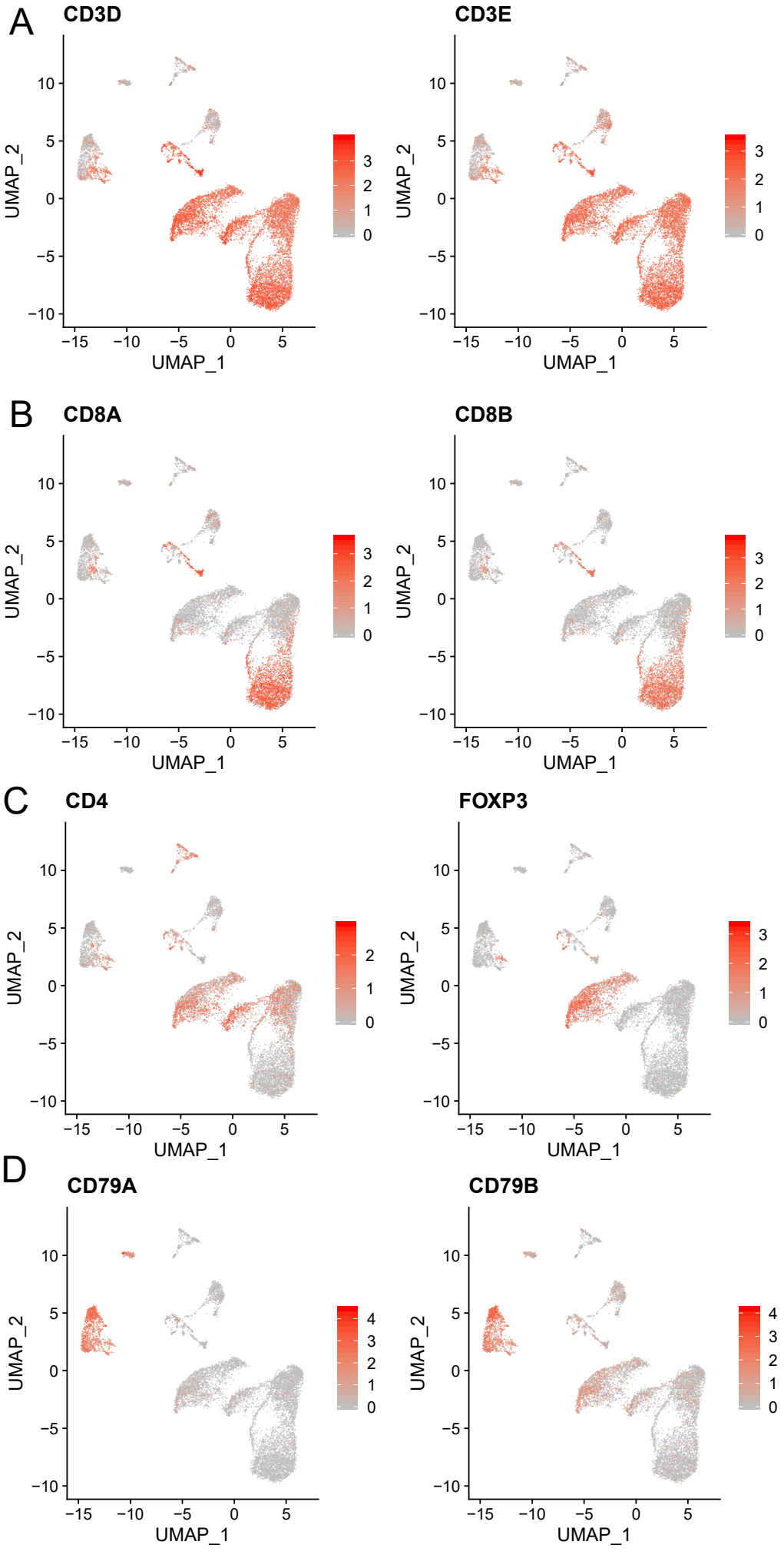


Figure S9

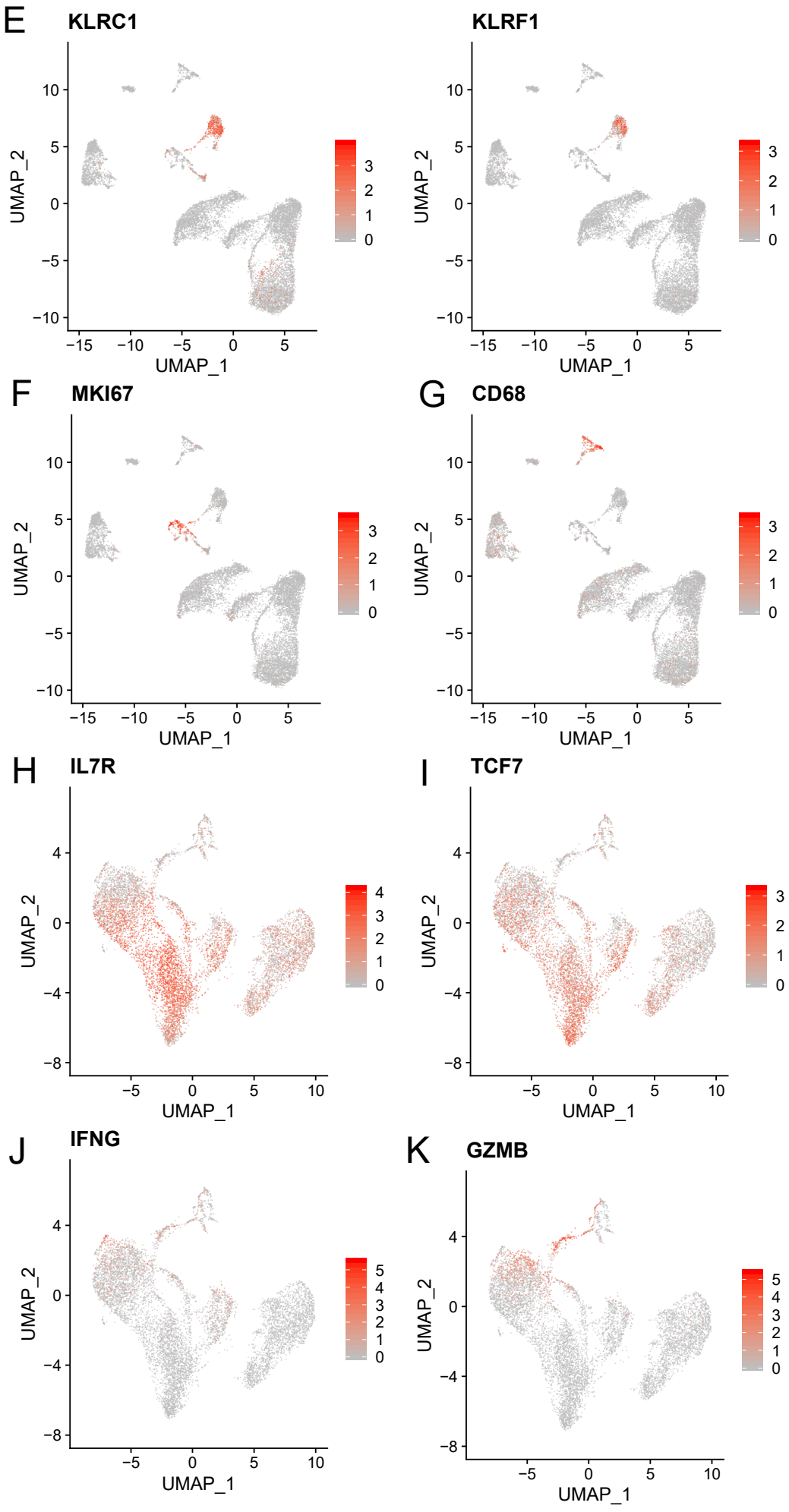
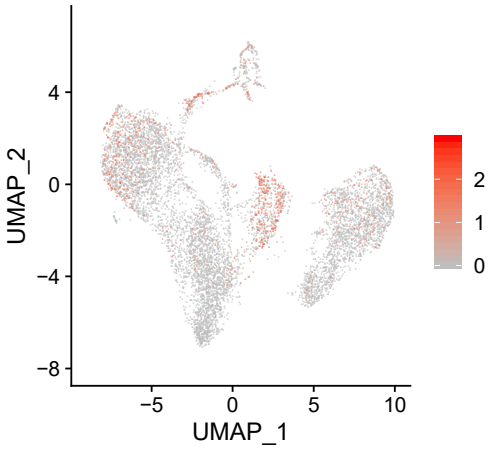
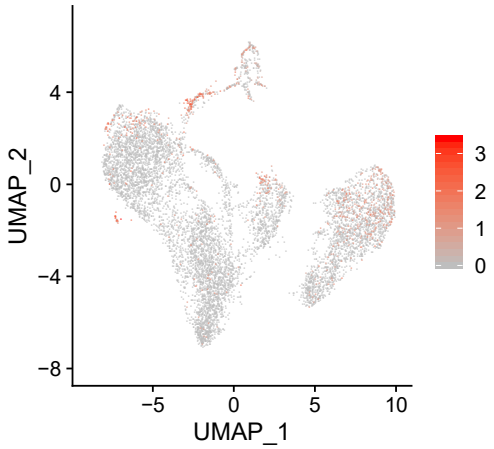

E
KLRC1
KLRF1
F
MKI67
G
CD68
H
IL7R
I
TCF7
J
IFNG
K
GZMB
UMAP_1
UMAP_2

Figure S9

L **PDCD1**



M **HAVCR2**



N **CTLA4**

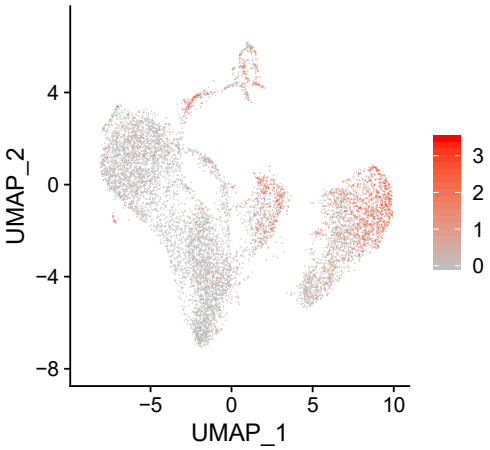
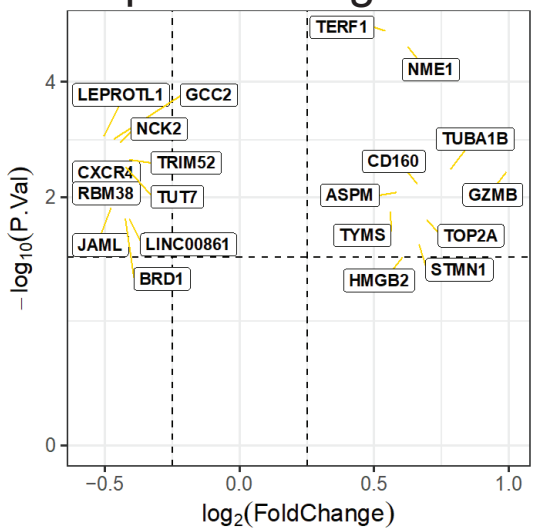


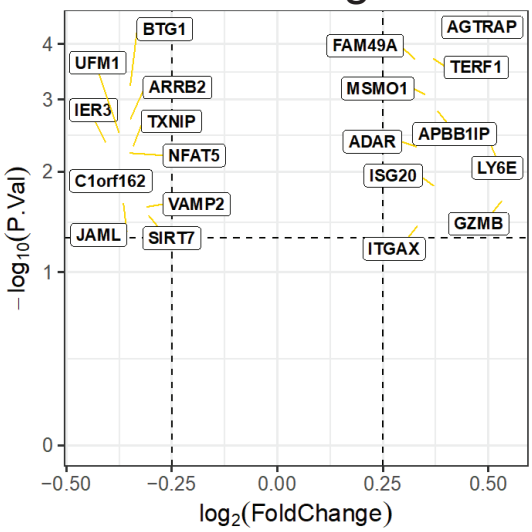
Figure S9. Single cell sequencing resolution of HGSC organoid co-cultures post treatment is high enough to differentiate all immune cell types: For pools of treated organoid co-cultures, all cells were analyzed for lineage and other marker expression. **A)** UMAP demonstrating *CD3D* and *CD3E* marker expression in CD8 T cells from pooled organoid co-cultures. **B)** UMAP demonstrating *CD8A* and *CD8B* marker expression in CD8 T cells from pooled organoid co-cultures. **C)** UMAP demonstrating *CD4* and *FOXP3* for CD4 T cell differentiation. **D)** UMAP demonstrating *CD79A* and *CD79B* for B cell differentiation. **E)** UMAP demonstrating *KLRC1* and *KLRF1* for NK cell differentiation. **F)** UMAP demonstrating *MKI67* expression to detect proliferating cells. **G)** UMAP demonstrating *CD68* expression to differentiate monocytes. **H and I)** UMAPs demonstrating naivety markers **H)** *IL7R* and **I)** *TCF7*. **J and K)** UMAPs demonstrating activation markers **J)** *IFNG* and **K)** *GZMB*. **L-N)** UMAPs demonstrating exhaustion markers **L)** *PDCD1*, **M)** *HAVCR2*, and **N)** *CTLA4*.

Figure S10

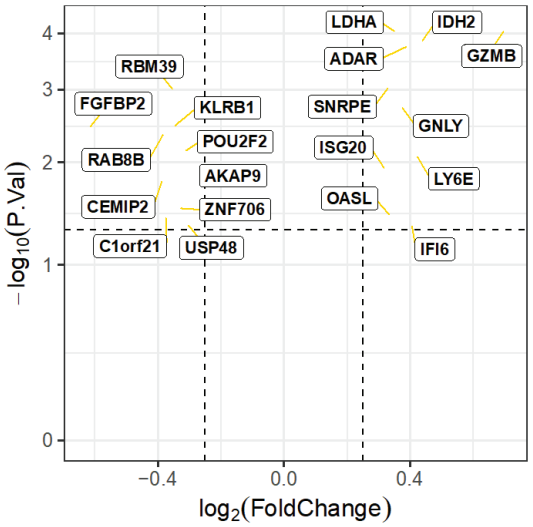
A



B



C



D

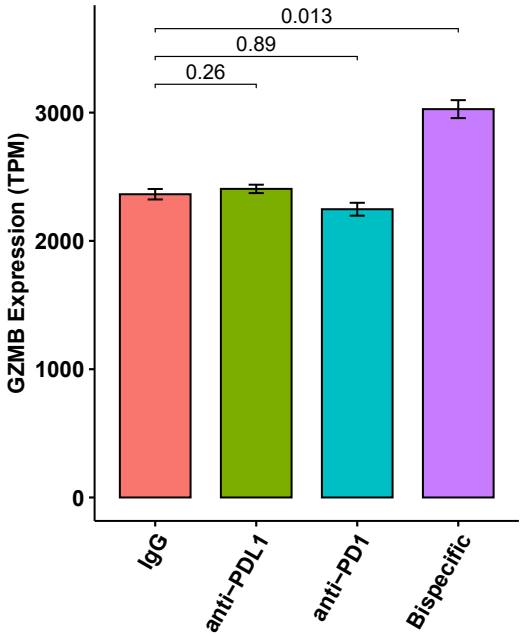


Figure S10. Differential expression analysis for NK cells comparing all treatments: Single cell RNA sequencing data was analyzed for differential expression between different treatment groups. **A)** Volcano plot showing differentially expressed genes for NK cells for IgG vs. Bispecific. **B)** Volcano plot showing differentially expressed genes for NK cells for IgG vs. PD-L1. **C)** Volcano plot showing differentially expressed genes for NK cells for IgG vs. PD-1. **D)** Bulk RNA sequencing analysis was performed on NK cells sorted from treated organoid co-cultures from a different tumor (20-35) than the one used for single cell RNA sequencing to verify the top hits. *GZMB* expression across each treatment is shown here as transcripts per million (TPM) with p-values on top of the bars generated using a one-tailed t-test and error bars representing standard deviation between two replicates.

Figure S11

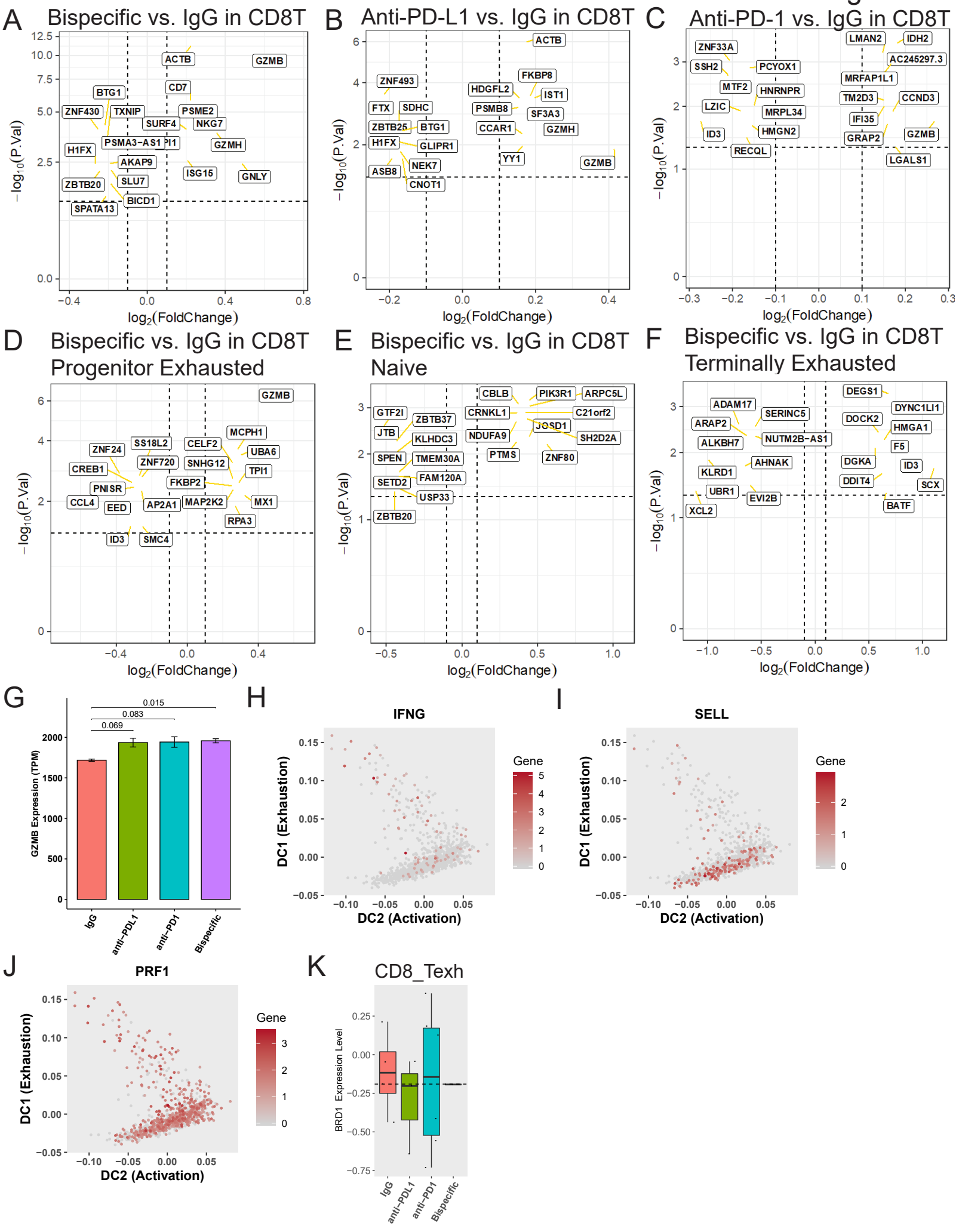

A
Bispecific vs. IgG in CD8T
B
Anti-PD-L1 vs. IgG in CD8T
C
Anti-PD-1 vs. IgG in CD8T
D
Bispecific vs. IgG in CD8T
Progenitor Exhausted
E
Bispecific vs. IgG in CD8T
Naive
F
Bispecific vs. IgG in CD8T
Terminally Exhausted
log2(FoldChange)
−log10(P.Val)
G
GZMB Expression (TPM)
0.015
0.083
0.069
IgG
anti-PDL1
anti-PD1
Bispecific
H
IFNG
I
SELL
DC1 (Exhaustion)
DC2 (Activation)
Gene
J
PRF1
K
CD8_Texh
BRD1 Expression Level

Figure S11. Differential expression in multiple CD8 T cell groups across treatments in HGSC organoid co-cultures: Single cell RNA sequencing data was analyzed for differential expression between different treatment groups. **A)** Volcano plot showing differentially expressed genes for all CD8 T cells for Bispecific antibody vs. IgG. **B)** Volcano plot showing differentially expressed genes for all CD8 T cells for anti-PD-L1 antibody vs. IgG. **C)** Volcano plot showing differentially expressed genes for all CD8 T cells for anti-PD-1 antibody vs. IgG. **D)** Volcano plot showing differentially expressed genes for progenitor exhausted CD8 T cells for Bispecific antibody vs. IgG. **E)** Volcano plot showing differentially expressed genes for naive CD8 T cells for Bispecific antibody vs. IgG. **F)** Volcano plot showing differentially expressed genes for terminally exhausted CD8 T cells for Bispecific antibody vs. IgG. **G)** Bulk RNA sequencing analysis was performed on bulk CD8 T cells sorted from treated organoid co-cultures from a different tumor (20-35) than the one used for single cell RNA sequencing to verify the top hits. *GZMB* expression across each treatment is shown here as transcripts per million (TPM) with p-values on top of the bars generated using a one-tailed t-test and error bars representing standard deviation between two replicates. **H-J)** Diffusion maps corresponding to Figure 5 pseudotime analysis for naïve, progenitor exhausted, and terminally exhausted CD8 T cells for **(H)** *IFNG*, **(I)** *SELL*, and **(J)** *PRF1*. The color code for gene expression level is shown on the right. **K)** Differential expression of BRD1 in terminally exhausted CD8 T cells (CD8_Texh) for which there were not enough cells in the bispecific group to analyze for anti-PD-1, anti-PD-L1, and the bispecific antibody compared to the isotype control.

Figure S12

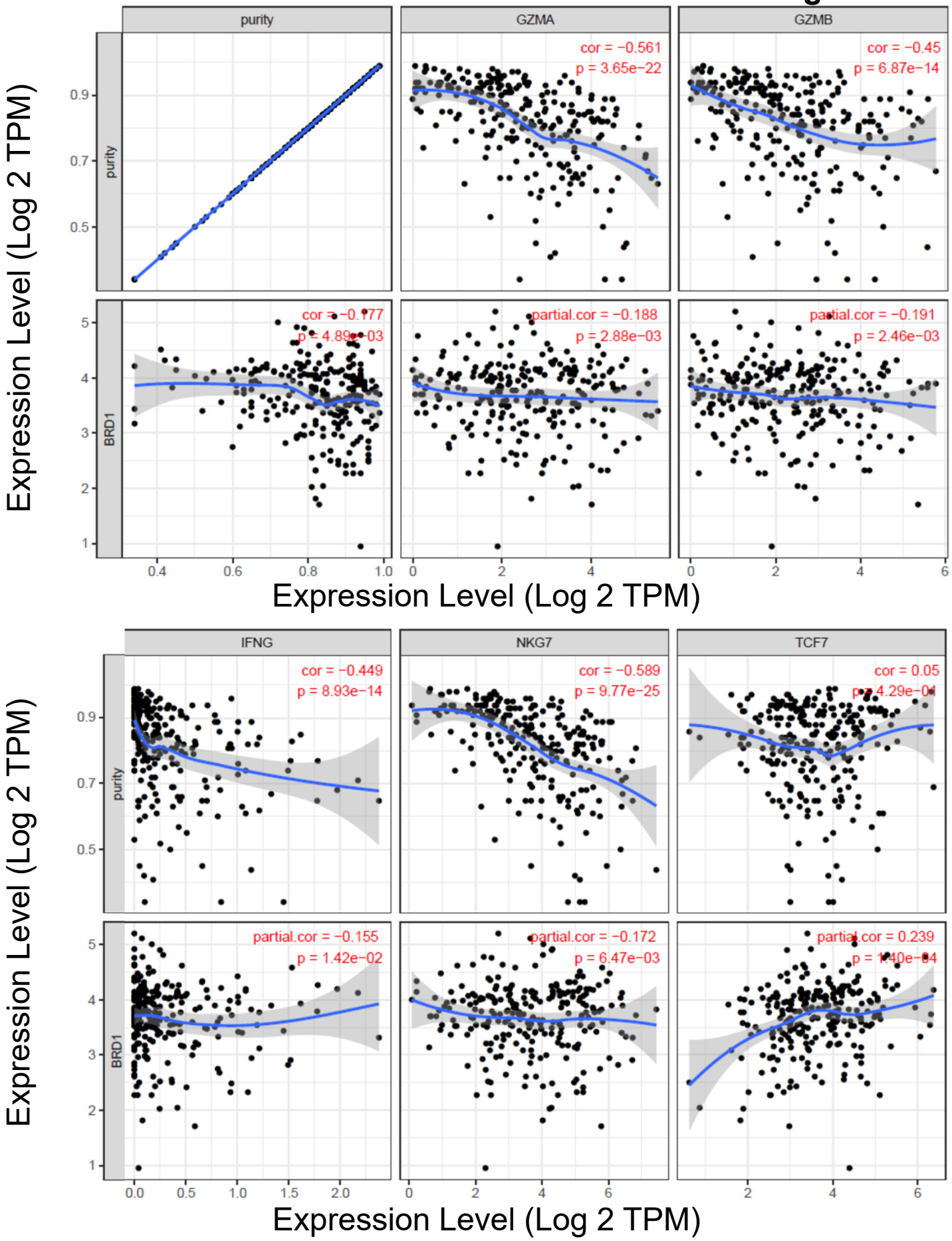
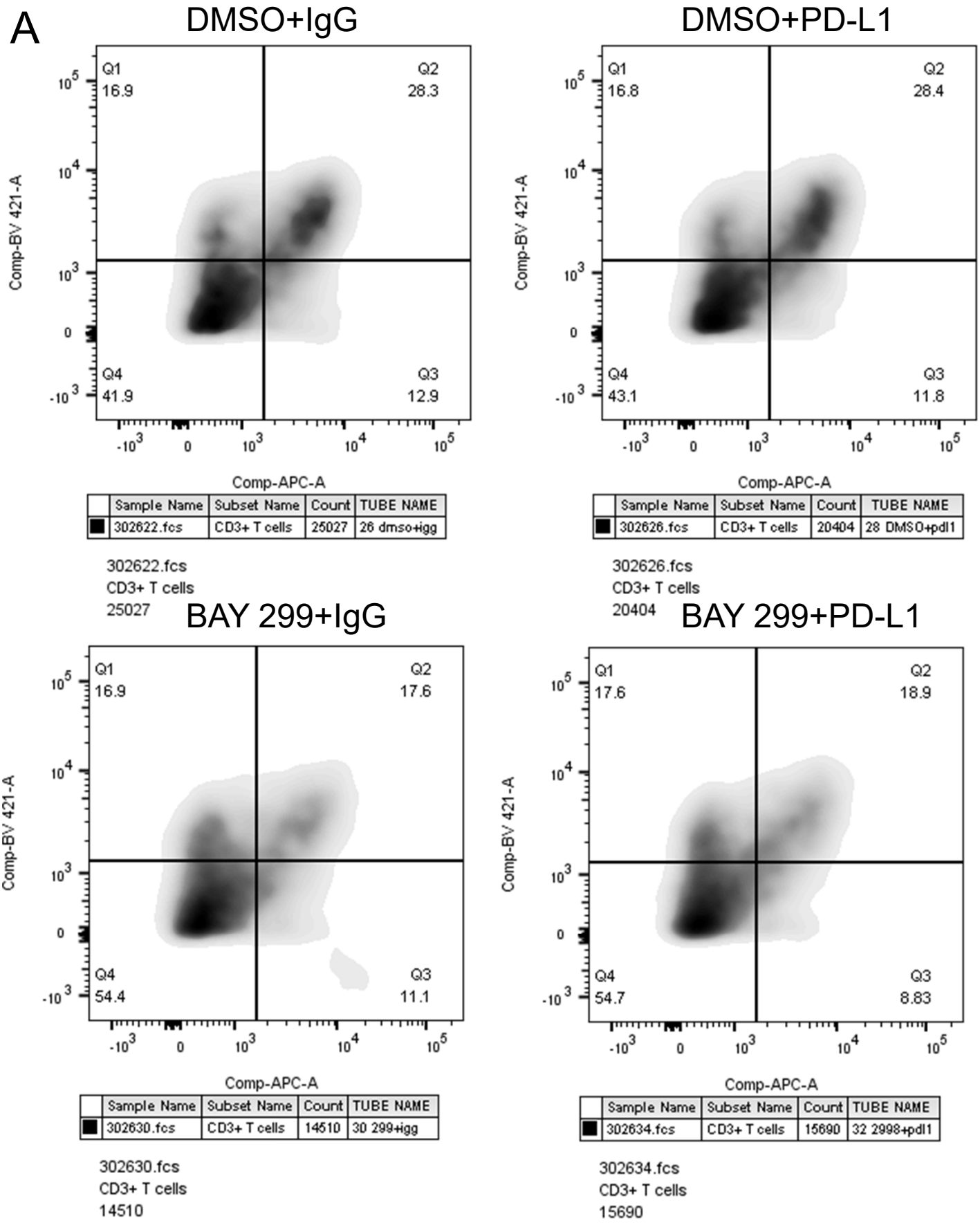

purity
GZMA
GZMB
IFNG
NKG7
TCF7
BRD1
cor = −0.561
p = 3.65e−22
cor = −0.45
p = 6.87e−14
cor = −0.177
p = 4.89e−03
partial.cor = −0.188
p = 2.88e−03
partial.cor = −0.191
p = 2.46e−03
cor = −0.449
p = 8.93e−14
cor = −0.589
p = 9.77e−25
cor = 0.05
p = 4.29e−01
partial.cor = −0.155
p = 1.42e−02
partial.cor = −0.172
p = 6.47e−03
partial.cor = 0.239
p = 1.40e−04
Expression Level (Log 2 TPM)
Expression Level (Log 2 TPM)
Expression Level (Log 2 TPM)
Expression Level (Log 2 TPM)

Figure S12. TIMER analysis of *BRD1* expression in intra-tumoral immune cells compared to activity and naivety markers: TIMER was used to analyze the up or downregulation of activation markers (Granzyme A=*GZMA*, Granzyme B=*GZMB*, *NKG7*, *IFNG*) and T cell naivety marker *TCF7* in the setting of *BRD1* expression in the intra-tumoral immune cells. The correlation and p values for each marker along with plots of log2 fold change of transcripts per million (TPM) with *BRD1* on the Y axis and the marker of interest on the X axis are shown without tumor purity correction (top in each panel) and with tumor purity correction (bottom in each panel).

CD3 T cells **Figure S13**

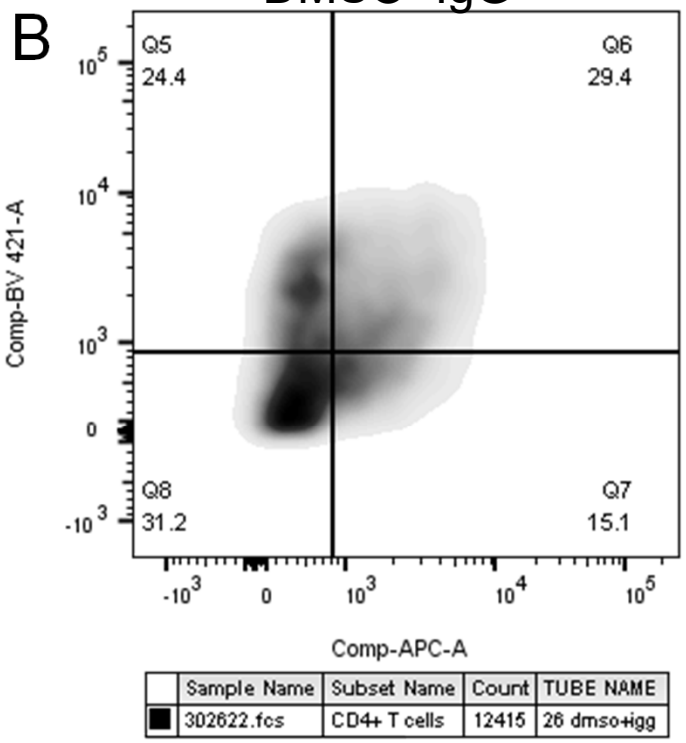
A



CD4 T cells **Figure S13**

B

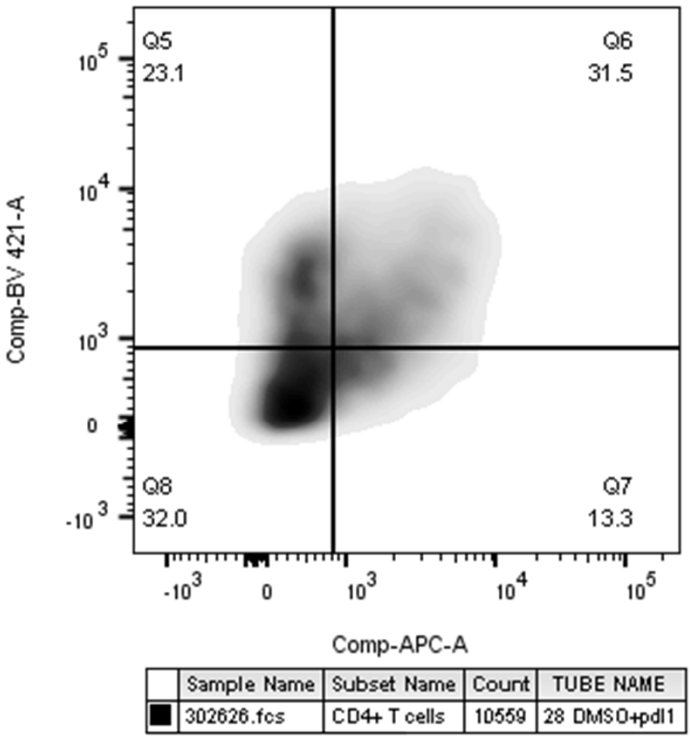
DMSO+IgG



	Sample Name	Subset Name	Count	TUBE NAME
■	302622.fcs	CD4+ T cells	12415	26 dmso+igg

302622.fcs
CD4+ T cells
12415

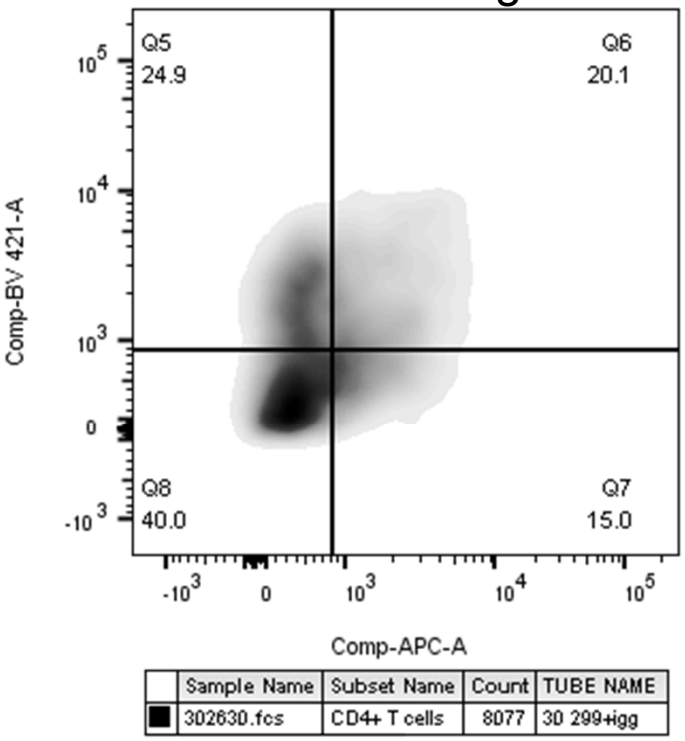
DMSO+PD-L1



	Sample Name	Subset Name	Count	TUBE NAME
■	302626.fcs	CD4+ T cells	10559	28 DMSO+pdl1

302626.fcs
CD4+ T cells
10559

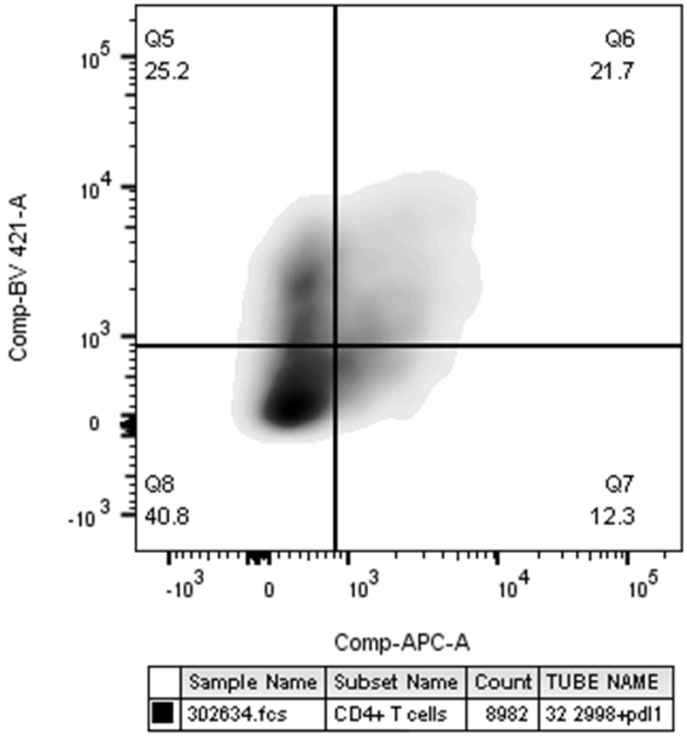
BAY 299+IgG



	Sample Name	Subset Name	Count	TUBE NAME
■	302630.fcs	CD4+ T cells	8077	30 299+igg

302630.fcs
CD4+ T cells
8077

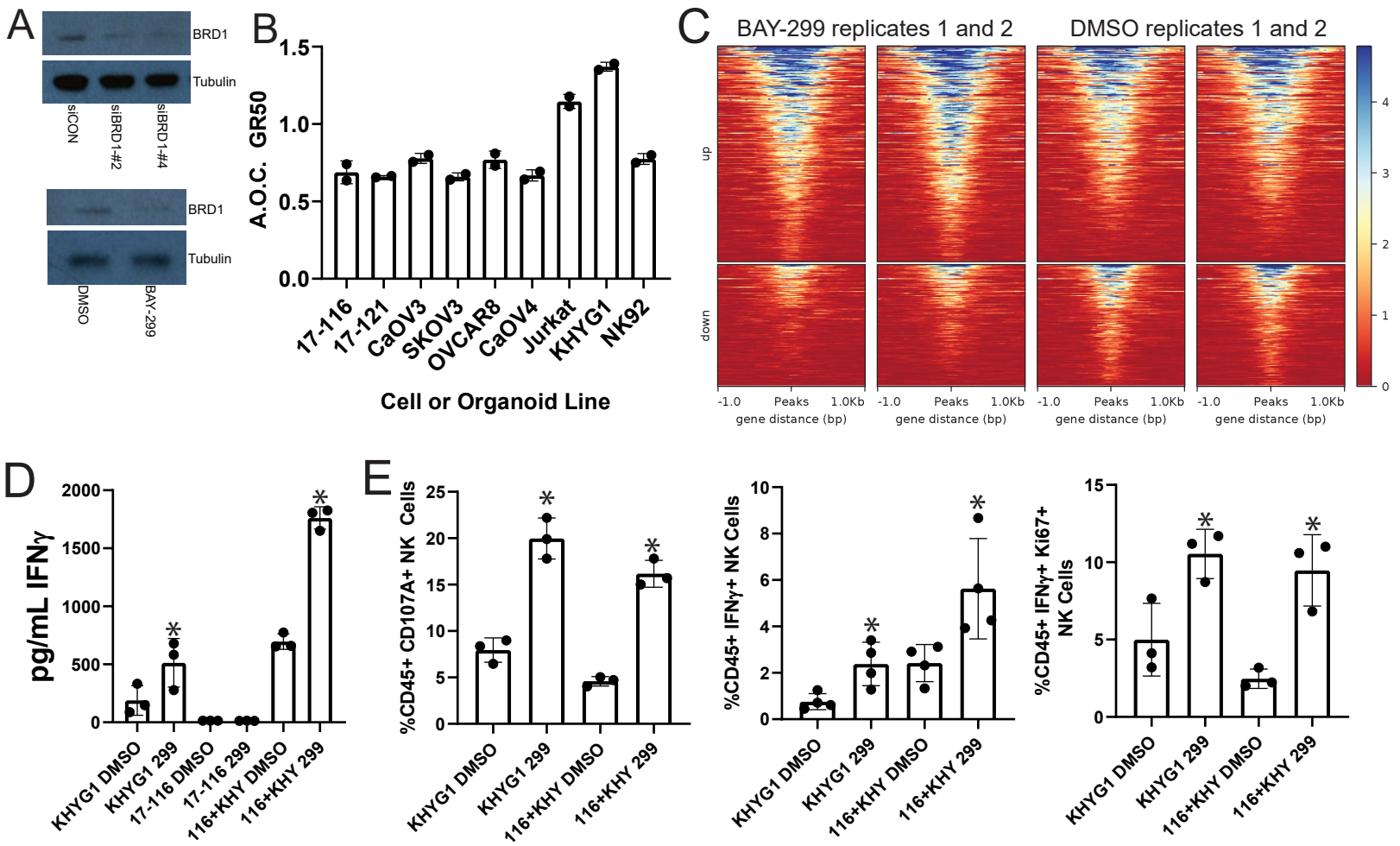
BAY 299+PD-L1



	Sample Name	Subset Name	Count	TUBE NAME
■	302634.fcs	CD4+ T cells	8982	32 2998+pdl1

302634.fcs
CD4+ T cells
8982

Figure S13. Quadrant analysis of TIM3 single, PD-1 single, TIM3/PD-1 double positive, and TIM3/PD-1 double negative cells after BAY-299 treatment: Organoid co-cultures from patient 20-35 were treated with either Isotype control, anti-PD-L1, anti-PD-1, or the bispecific antibody combined with either DMSO or BAY-299 and assessed by flow cytometry for TIM3/PD-1 double positive immune cells. The quadrant gates for TIM3 single, PD-1 single, TIM3/PD-1 double positive, and TIM3/PD-1 double negative **(A)** CD3 and **(B)** CD4 cells as a percent of CD45+ cells are shown here for the IgG and anti-PD-L1 combination treated organoids. In each graph PD-1 is the Y axis and TIM3 is the X axis. Only IgG and anti-PD-L1 data is shown here because the anti-PD-1 and bispecific antibodies compete with the PD-1 flow cytometry antibody. Percentages of each population are shown in the corner of each quadrant. In **A)**, the percentage of PD-1 (Q1) and TIM3 (Q3) single positive and double positive (Q2) CD3 T cells is shown on the bottom as a percent of CD45 positive cells. In **B)**, the percentage of PD-1 (Q5) and TIM3 (Q7) single positive and double positive (Q6) CD4 T cells is shown on the bottom as a percent of CD45 positive cells.


A
BRD1
Tubulin
siCON
siBRD1#2
siBRD1#4
BRD1
Tubulin
DMSO
BAY-299
B
A.O.C. GR50
1.5
1.0
0.5
0.0
17-116
17-121
CaOV3
SKOV3
OVCAR8
CaOV4
Jurkat
KHYG1
NK92
Cell or Organoid Line
C
BAY-299 replicates 1 and 2
DMSO replicates 1 and 2
up
down
-1.0
Peaks
1.0Kb
gene distance (bp)
4
3
2
1
0

D
pg/mL IFNγ
2000
1500
1000
500
0
KHYG1 DMSO
KHYG1 299
17-116 DMSO
17-116 299
116+KHY DMSO
116+KHY 299
E
%CD45+ CD107A+ NK Cells
25
20
15
10
5
0
KHYG1 DMSO
KHYG1 299
116+KHY DMSO
116+KHY 299
%CD45+ IFNγ+ NK Cells
10
8
6
4
2
0
KHYG1 DMSO
KHYG1 299
116+KHY DMSO
116+KHY 299
%CD45+ IFNγ+ Ki67+ NK Cells
15
10
5
0
KHYG1 DMSO
KHYG1 299
116+KHY DMSO
116+KHY 299

Figure S14. BAY-299 effects *in vitro*. A) The NK cell line KHYG1 was either transfected with control or two distinct *BRD1* specific siRNAs (to validate the BRD1 antibody) or treated with vehicle or BAY-299. Western blots of the differently treated cells are shown here for BRD1 and Tubulin as a loading control. **B)** Two HGSC organoid cultures (17-116 and 17-121), four HGSC cell lines (SKOV3, OVCAR8, CaOV3, and CaOV4), a T cell line (Jurkat) and two NK cell lines (NK92 and KHYG1) were treated with a dose range of BAY-299 and analyzed for cytotoxicity. The growth rate corrected area over the curve (A.O.C. GR50) is shown here. The larger the area, the more sensitive the line is to BAY-299. Two replicates were performed per cell/organoid line. Error bars represent standard deviation. **C)** Heatmaps of chromatin accessibility for all sites are shown for the ATAC-seq data in Figure 7A for the two BAY-299 replicates (left) and the two DMSO replicates (right). The scale is shown on the right. **D)** The KHYG1 NK cell line (KHY) was treated with either DMSO or BAY-299 (299) and plated either alone or in combination with the HGSC organoid line 17-116 (116). Supernatants were analyzed for IFNγ by ELISA. The results are shown here as pg/mL in triplicate with error bars representing standard deviation. **E)** The KHYG1 NK cell line (KHY) was treated with either DMSO or BAY-299 (299) and plated either alone or in combination with the HGSC organoid line 17-116 (116). NK cells were analyzed by flow cytometry for CD107A expression (left), IFNγ expression (middle), and IFNγ and Ki67 co-expression (right). Bar graphs show the average of three-four experiments with error bars representing standard deviation. *=$p<0.05$ using a paired t-test.

Figure S15

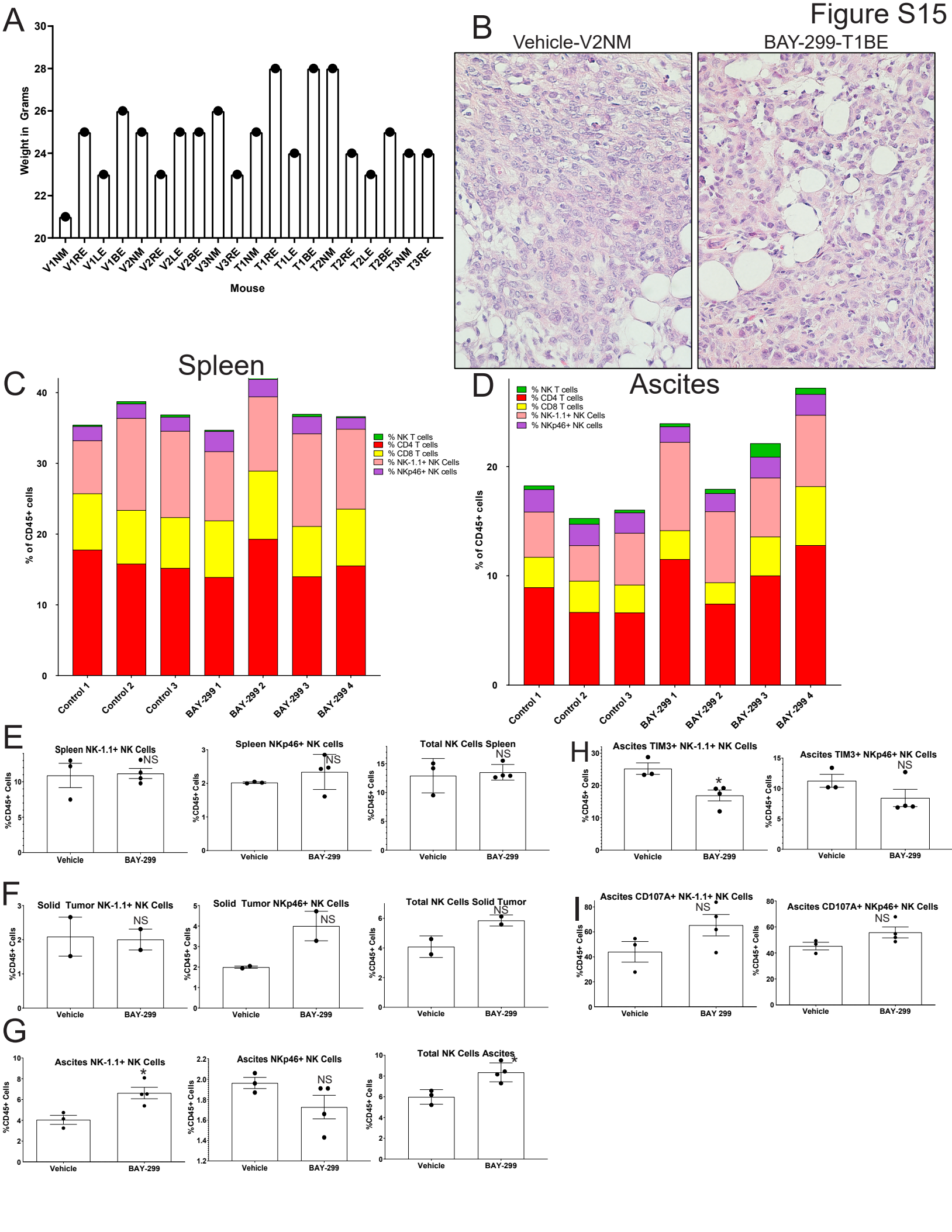

A
Weight in Grams
Mouse
V1NM V1RE V1LE V1BE V2NM V2RE V2LE V2BE V3NM V3RE T1NM T1RE T1LE T1BE T2NM T2RE T2LE T2BE T3NM T3RE
B
Vehicle-V2NM
BAY-299-T1BE
C
Spleen
% of CD45+ cells
% NK T cells
% CD4 T cells
% CD8 T cells
% NK-1.1+ NK Cells
% NKp46+ NK cells
Control 1 Control 2 Control 3 BAY-299 1 BAY-299 2 BAY-299 3 BAY-299 4
D
Ascites
E
Spleen NK-1.1+ NK Cells
Spleen NKp46+ NK cells
Total NK Cells Spleen
%CD45+ Cells
Vehicle BAY-299
NS
H
Ascites TIM3+ NK-1.1+ NK Cells
Ascites TIM3+ NKp46+ NK Cells
F
Solid Tumor NK-1.1+ NK Cells
Solid Tumor NKp46+ NK Cells
Total NK Cells Solid Tumor
I
Ascites CD107A+ NK-1.1+ NK Cells
Ascites CD107A+ NKp46+ NK Cells
BAY 299
G
Ascites NK-1.1+ NK Cells
Ascites NKp46+ NK Cells
Total NK Cells Ascites
*

Figure S15. Effects of BAY-299 *in vivo*. A) The animal weights for each individual animal on the final day of the experiment are shown here with V signifying vehicle and T signifying treatment in the animal names on the X axis. **B)** Hematoxylin and Eosin stains of a representative solid tumor from the vehicle (left) and treatment (right) groups. **C)** Spleens were harvested from each animal in both the vehicle and treatment groups. For vehicle, the spleens of 3 or 4 animals were combined, and for treatment 3 groups of three spleens were combined and one individual spleen was separate (BAY-299 4). The single cell suspensions of the spleens were analyzed for T and NK composition which is shown here. Each color represents a cell type and each bar represents a group or an animal. **D)** Ascites was harvested from each animal in both the vehicle and treatment groups. For vehicle, the ascites of 3 or 4 animals were combined, and for treatment 3 groups of three ascites were combined and one individual ascites sample was separate (BAY-299 4). The single cell suspensions of the ascites was analyzed for T and NK composition which is shown here. Each color represents a cell type and each bar represents a group or an animal. **E, F, G)** NK cell composition was analyzed for the **(E)** spleen, **(F)** solid tumors, and **(G)** ascites for the different component treatment pools. Percentage of NK-1.1+ NK cells (left), NKp46+ NK cells (middle), and combined NK-1.1 and NKp46+ NK cells (right) is shown here for each compartment as a percent of CD45+ cells. Error bars represent standard deviation. **H)** TIM3 expression was analyzed in both treatment groups on NK-1.1+ NK cells (left) and NKp46+ NK cells (right) in the ascites and the percent of TIM3+ cells for each treatment group is shown here as a percentage of CD45+ cells for the ascites. Error bars represent standard deviation. **I)** CD107A expression on NK cells was analyzed for all compartments. Here the CD107A expression on NK-1.1 (left) or NKp46+ (right) NK cells is shown for the ascites. Error bars represent standard deviation. NS=Not significant. *$p<0.05$ by an unpaired t-test.

SUPPLEMENTARY TABLE LEGENDS

Table S1. Differential expression analysis for CD8 T cells: For each table the first column is the gene name, the second the p-value for that gene, the third the average log fold change, the fourth the percentile 1, the fifth the percentile 2, the sixth the p value adjustment, the seventh the Gene ID, and the eighth the log of the p-value. Clusters are mapped in Figure 3E. **A)** Differential expression analysis for cluster 1 CD8 T cells comparing IgG vs PD-1. **B)** Differential expression analysis for cluster 1 CD8 T cells comparing IgG vs. PD-L1. **C)** Differential expression analysis for cluster 1 CD8 T cells comparing IgG vs. Bispecific. **D)** Differential expression analysis for cluster 1 CD8 T cells comparing PD-1 vs. Bispecific. **E)** Differential expression analysis for cluster 1 CD8 T cells comparing PD-L1 vs. Bispecific. **F)** Differential expression analysis for cluster 13 CD8 T cells comparing IgG vs. PD-1. **G)** Differential expression analysis for cluster 13 CD8 T cells comparing IgG vs. PD-L1. **H)** Differential expression analysis for cluster 13 CD8 T cells comparing IgG vs. Bispecific. **I)** Differential expression analysis for cluster 13 CD8 T cells comparing PD-1 vs. Bispecific. **J)** Differential expression analysis for cluster 13 CD8 T cells PD-L1 vs. Bispecific.

Table S2. Differential expression analysis for Treg CD4 T cells For each table the first column is the gene name, the second the p-value for that gene, the third the average log fold change, the fourth the percentile 1, the fifth the percentile 2, the sixth the p value adjustment, the seventh the Gene ID, and the eighth the log of the p-value. Clusters are mapped in Figure 3E. **A)** Differential expression analysis for cluster 4 CD4 Treg cells comparing IgG vs PD-1. **B)** Differential expression analysis for cluster 4 CD4 Treg cells comparing IgG vs. PD-L1. **C)** Differential expression analysis for cluster 4 CD4 Treg cells comparing IgG vs. Bispecific. **D)** Differential expression analysis for cluster 4 CD4 Treg cells comparing PD-1 vs. Bispecific. **E)** Differential expression analysis for cluster 4 CD4 Treg cells comparing PD-L1 vs. Bispecific. **F)** Differential expression analysis for cluster 7 and 8 CD4 Treg cells comparing IgG vs. PD-1. **G)**

Differential expression analysis for cluster 7 and 8 CD4 Treg cells comparing IgG vs. PD-L1. **H)** Differential expression analysis for cluster 7 and 8 CD4 Treg cells comparing IgG vs. Bispecific. **I)** Differential expression analysis for cluster 7 and 8 CD4 Treg cells comparing PD-1 vs. Bispecific. **J)** Differential expression analysis for cluster 7 and 8 CD4 Treg cells PD-L1 vs. Bispecific.

Table S3. Differential expression analysis for non-Treg CD4 T cells: For each table the first column is the gene name, the second the p-value for that gene, the third the average log fold change, the fourth the percentile 1, the fifth the percentile 2, the sixth the p value adjustment, the seventh the Gene ID, and the eighth the log of the p-value. Clusters are mapped in Figure 3E. **A)** Differential expression analysis for CD4+PD-1+FOXP3- T cells comparing IgG vs PD-1. **B)** Differential expression analysis for CD4+PD-1+FOXP3- T cells comparing IgG vs. PD-L1. **C)** Differential expression analysis for CD4+PD-1+FOXP3- T cells comparing IgG vs. Bispecific. **D)** Differential expression analysis for CD4+PD-1+FOXP3- T cells comparing PD-1 vs. Bispecific. **E)** Differential expression analysis for CD4+PD-1+FOXP3- T cells comparing PD-L1 vs. Bispecific. **F)** Differential expression analysis CD4+PD-1-FOXP3- T cells comparing IgG vs. PD-1. **G)** Differential expression analysis for CD4+PD-1-FOXP3- T cells comparing IgG vs. PD-L1. **H)** Differential expression analysis for CD4+PD-1-FOXP3- T cells comparing IgG vs. Bispecific. **I)** Differential expression analysis for CD4+PD-1-FOXP3- T cells comparing PD-1 vs. Bispecific. **J)** Differential expression analysis for CD4+PD-1-FOXP3- T cells PD-L1 vs. Bispecific.

Table S4. Differential expression analysis for NK cells: For each table the first column is the gene name, the second the p-value for that gene, the third the average log fold change, the fourth the percentile 1, the fifth the percentile 2, the sixth the p value adjustment, the seventh the Gene ID, and the eighth the log of the p-value. **A)** Differential expression analysis for NK cells comparing IgG vs PD-1. **B)** Differential expression analysis for NK cells comparing IgG vs.

PD-L1. **C)** Differential expression analysis for NK cells comparing IgG vs. Bispecific. **D)** Differential expression analysis for NK cells comparing PD-1 vs. Bispecific. **E)** Differential expression analysis for NK cells comparing PD-L1 vs. Bispecific.

Table S5. List of Genes Used to Generate NK Cell Activation Scores: List of 22 genes queried to generate NK cell activation scores for main text Figure 4A.