Supplementary Information for

Dual checkpoint blockade of CD47 and PD-L1 using an affinity-tuned bispecific antibody maximizes anti-tumor immunity

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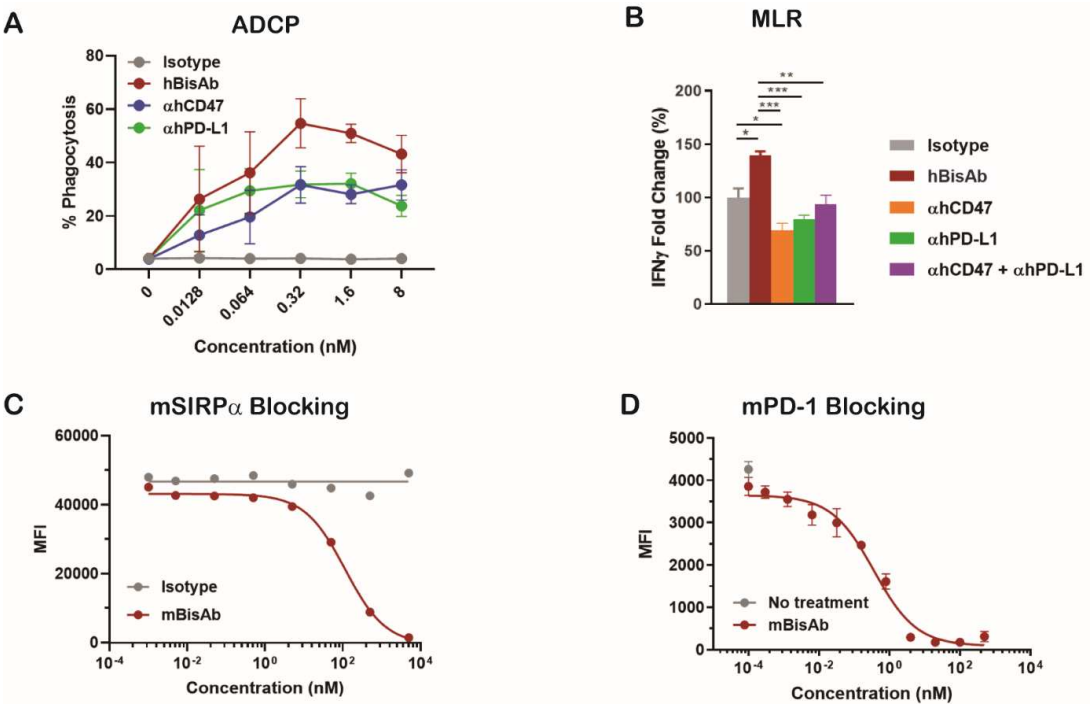
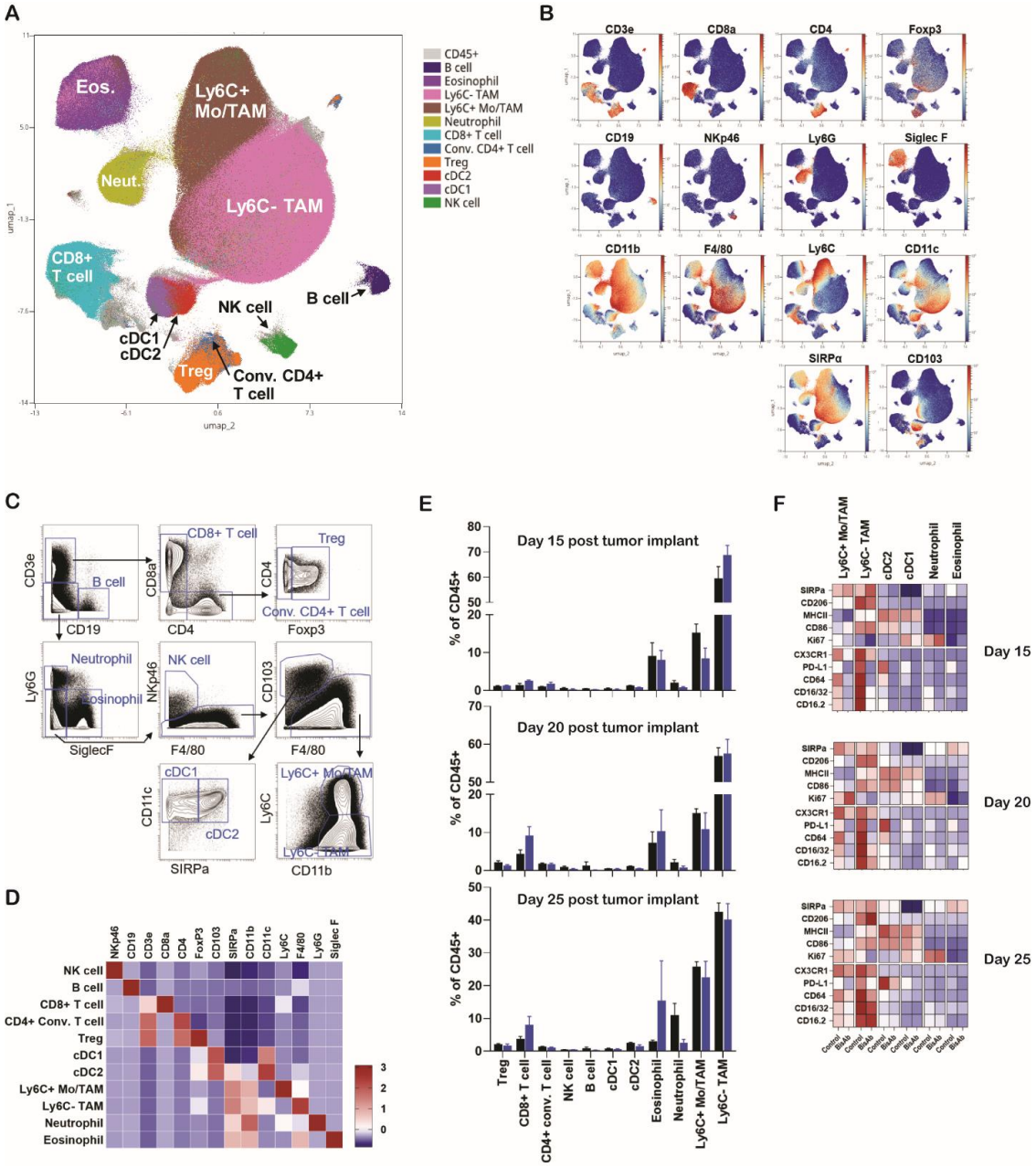


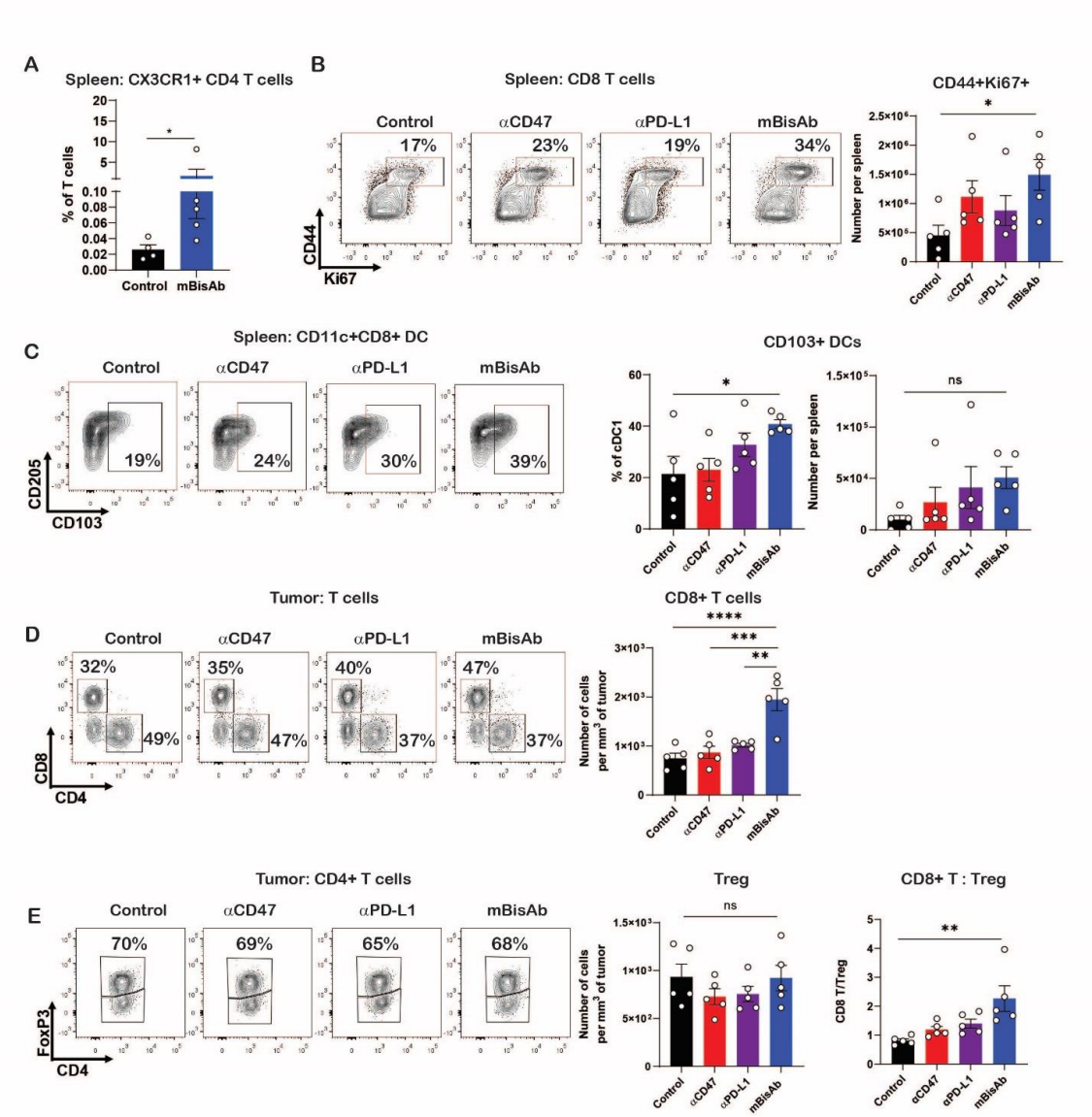
Supplementary Figure 1. ***In vitro*** **functionality of hBisAb and mBisAb** (**A**) Phagocytosis of NCI-H292 tumor cells by human macrophages in the presence of human IgG1 isotype controls, hBisAb, αhCD47, and αhPD-L1. A mixture of CFSE labeled tumor cells and CTV labeled macrophages were co-cultured at a 1:2 ratio for 2 hours with increasing concentrations the antibodies and analyzed by flow cytometry. Phagocytosis of total tumor cells is represented as fold change compared to isotype treatment. (**B**) MLR was conducted to assess concentration of IFNγ at 120 hours in the supernatant by ELISA. A mixture of LPS matured DCs and purified $CD4^+$ T cells were co-cultured at a 1:4 ratio with 200 nM of isotype control, hBisAb, αhCD47, αhPD-L1, or combination treatment of αhCD47 and αhPD-L1 at same molar ratio. *p<0.05, **p<0.01, ***p<0.001, One-way ANOVA. (**C**) *In vitro* blocking activity of mBisAb on the mouse CD47/SIRPα interaction as measured by flow cytometry. (**D**) *In vitro* blocking activity of mBisAb on the mouse PD-L1/PD-1 interaction as measured by flow cytometry.



Supplementary Figure 2. Designation of intratumoral immune cell population by CyTOF

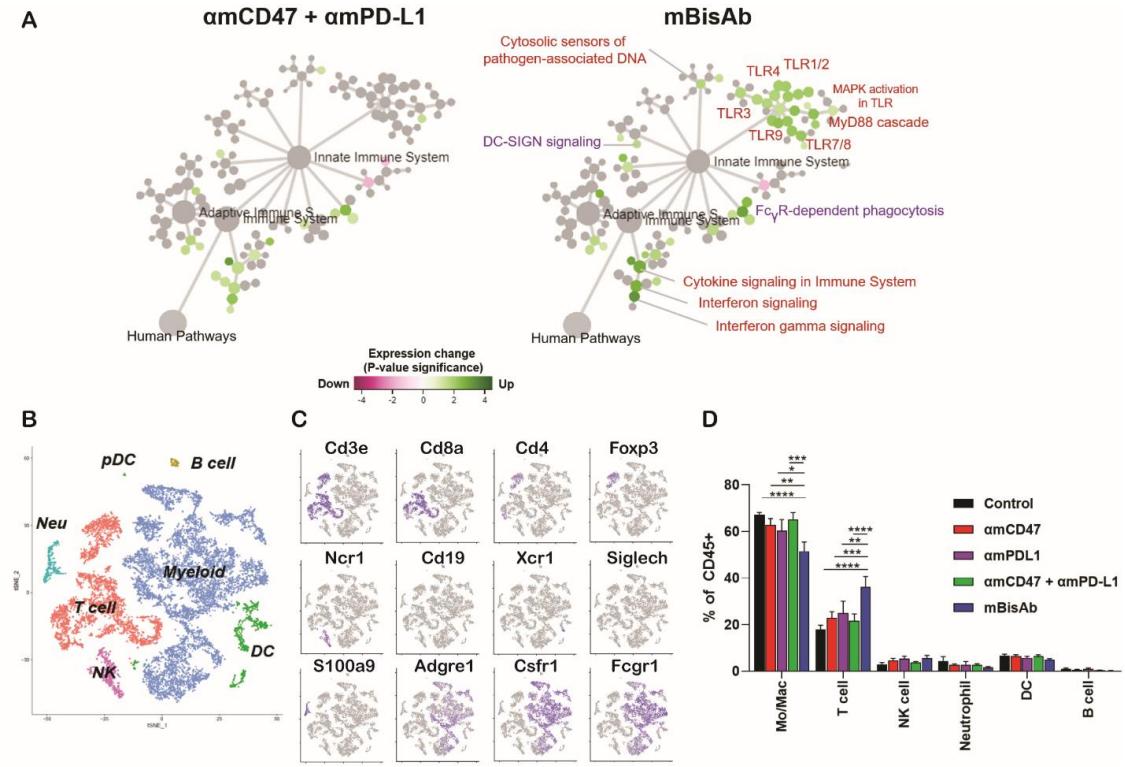
(**A-F**) The same samples of MC38 tumors and spleens in Figure 4A-F were further analyzed by CyTOF. (**A**) UMAP plot of total intratumoral CD45^{+} cells. Populations identified via biaxial gating are overlaid. (**B**) Single-cell expression of lineage markers used to define major leukocyte

populations. (**C**) Identification of major leukocyte populations via biaxial gating. (**D**) Relative expression of lineage markers within major leukocyte populations. (**E**) Frequency of leukocyte populations among total $CD45^+$ cells in control (black) or mBisAb-treated (blue) tumors at the indicated timepoints post-first dose of treatment. (**F**) Relative expression of activation markers within myeloid populations in control or mBisAb-treated tumors at the indicated timepoints post-tumor implantation.

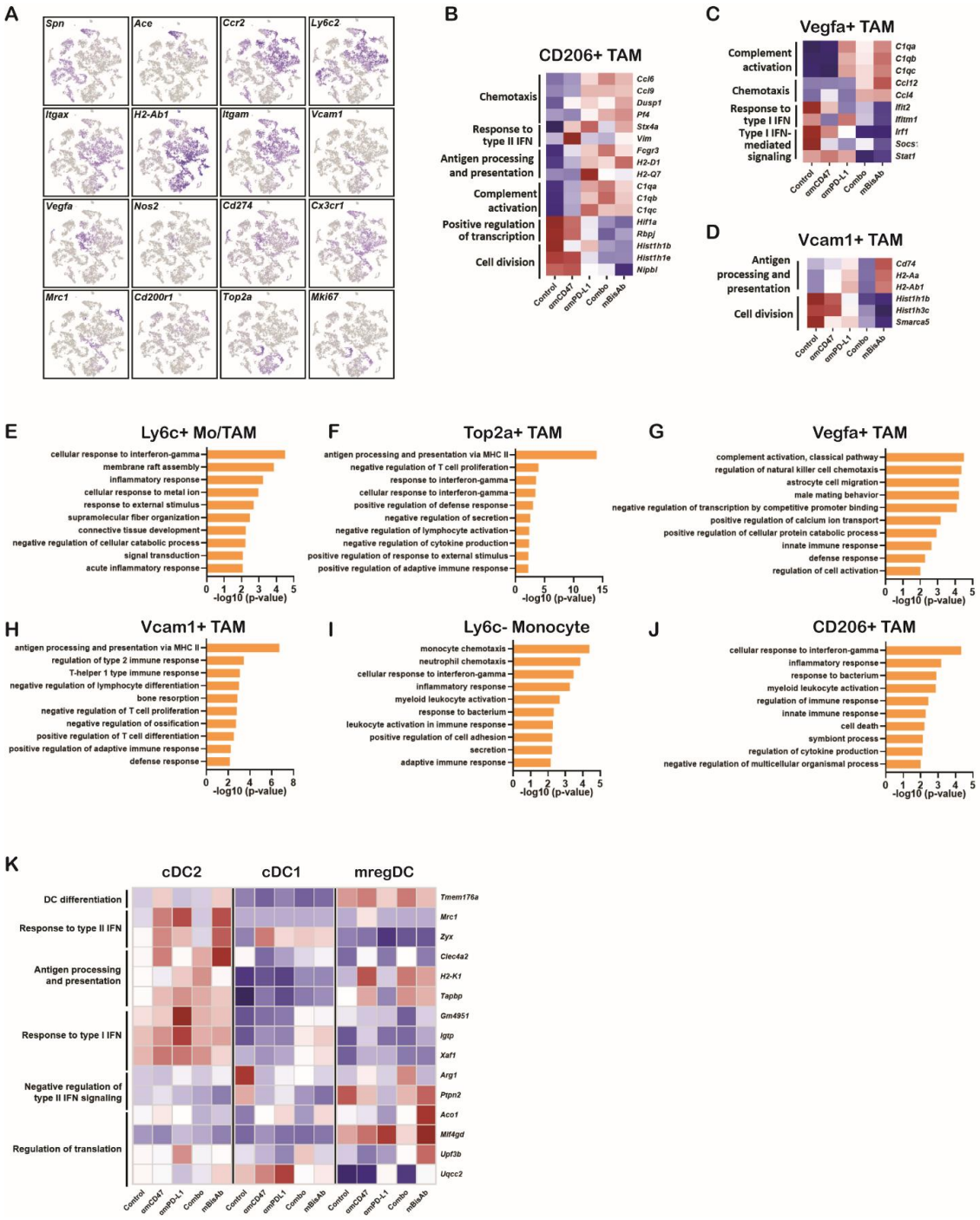


Supplementary Figure 3. Flow cytometric characterization of intratumoral and splenic immune populations in mBisAb-treated mice (**A**) Mice were treated as described in Figure 4. Frequency of $CD44^{hi}CD62L^{lo}CX3CR1^{+}PD\text{-}1^{+}FoxP3^{-}CD4^{+}$ T cells among total T cells in the spleens of control (black) or mBisAb-treated (blue) mice at day 20 post-implantation as determined by CyTOF analysis. *P<0.05, Mann Whitney test.(**B-E**) MC38-bearing mice were treated with isotype (control, black), αmCD47 (red), αmPD-L1(purple), or mBisAb (blue) every 3 to 4 days

starting at day 10 post-tumor implantation. Tumors and spleens were then harvested at day 20 for flow cytometric analysis (*n*=5 mice/group). (**B**) Quantification of activated proliferating ($CD44^+Ki67^+$) CD8 T cells in the spleens of mice with each indicated treatment, with representative flow cytometry plots shown to the left. (**C**) Frequency of $CD103^+$ cells within cDC1 and quantification of $CD103^+$ DCs in the spleens of mice with indicated treatments, with representative flow cytometry plots shown to the left. (**D**) Quantification of total CD8 T cells in the tumors of mice with the indicated treatments, with representative flow cytometry plots shown to the left. (**E**) Quantification of total Tregs ($CD4^+FoxP3^+$ T cells) in the tumors of mice with the indicated treatments, and ratio of the total number of $CD8^+$ T cells relative to the total number of Tregs in each tumor, with representative gating of Tregs shown to the left. ns P>0.05, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, One-way ANOVA with multiple comparisons.

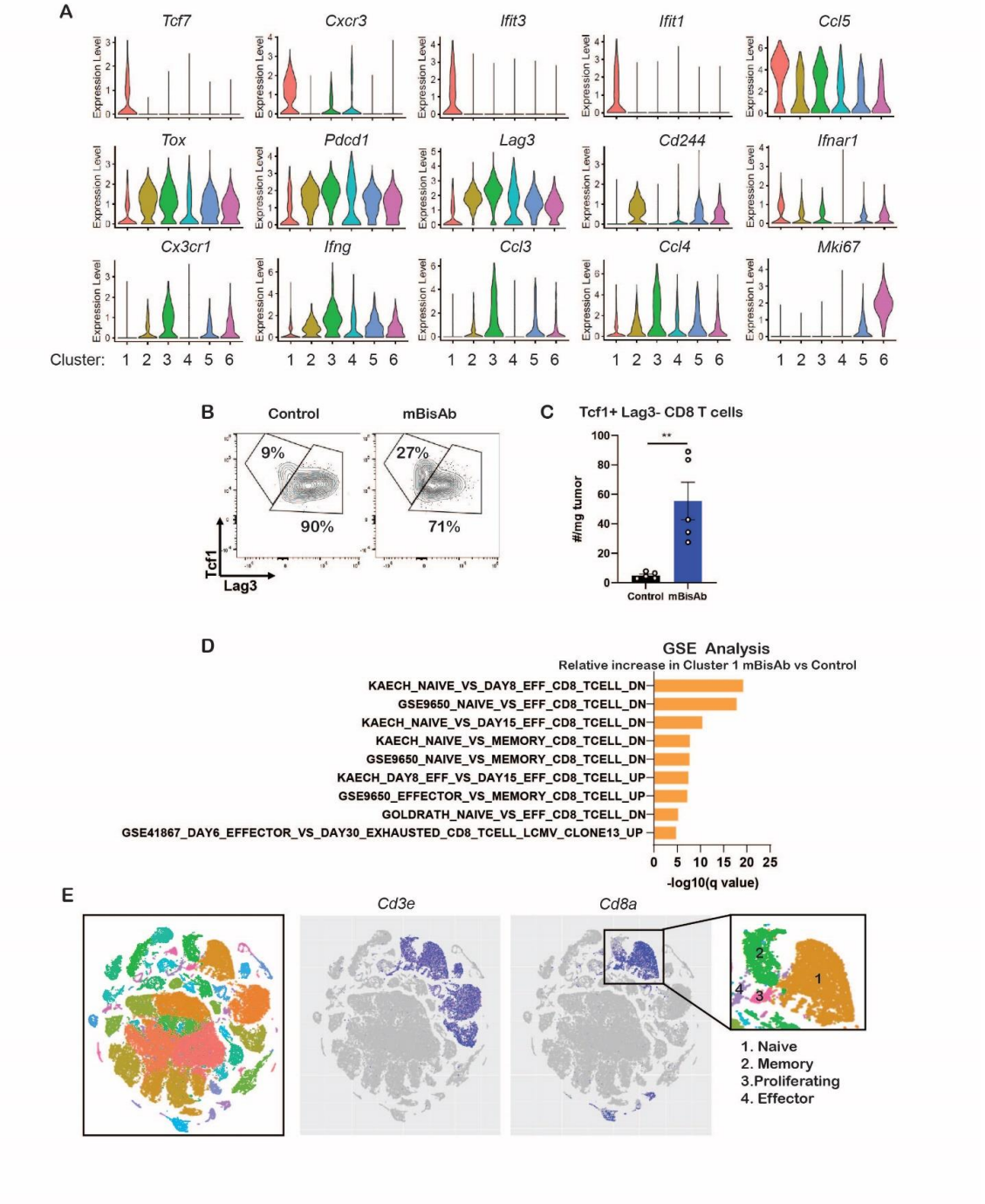


Supplementary Figure 4. Pathway analysis by Nanostring and designation of immune cell populations by scRNA-seq (**A**) Pathway correlation network analysis on the samples in mBisAb-treated group compared with the αmCD47 and αmPD-L1 combination group in Figure 5A. (**B-D**) Analysis of scRNA-seq samples in Figure 5. (**B**) tSNE analysis of intratumoral $CD45^+$ cells from across all treatment conditions, with cell lineages overlaid. (**C**) Single-cell expression of representative lineage markers used to designate major leukocyte populations. (**D**) Frequency of each lineage among total $CD45^+$ cells in each treatment condition. *P<0.05, **P<0.01, ***P<0.001, ***P<0.0001, two-way ANOVA with tukey's multiple comparison test.

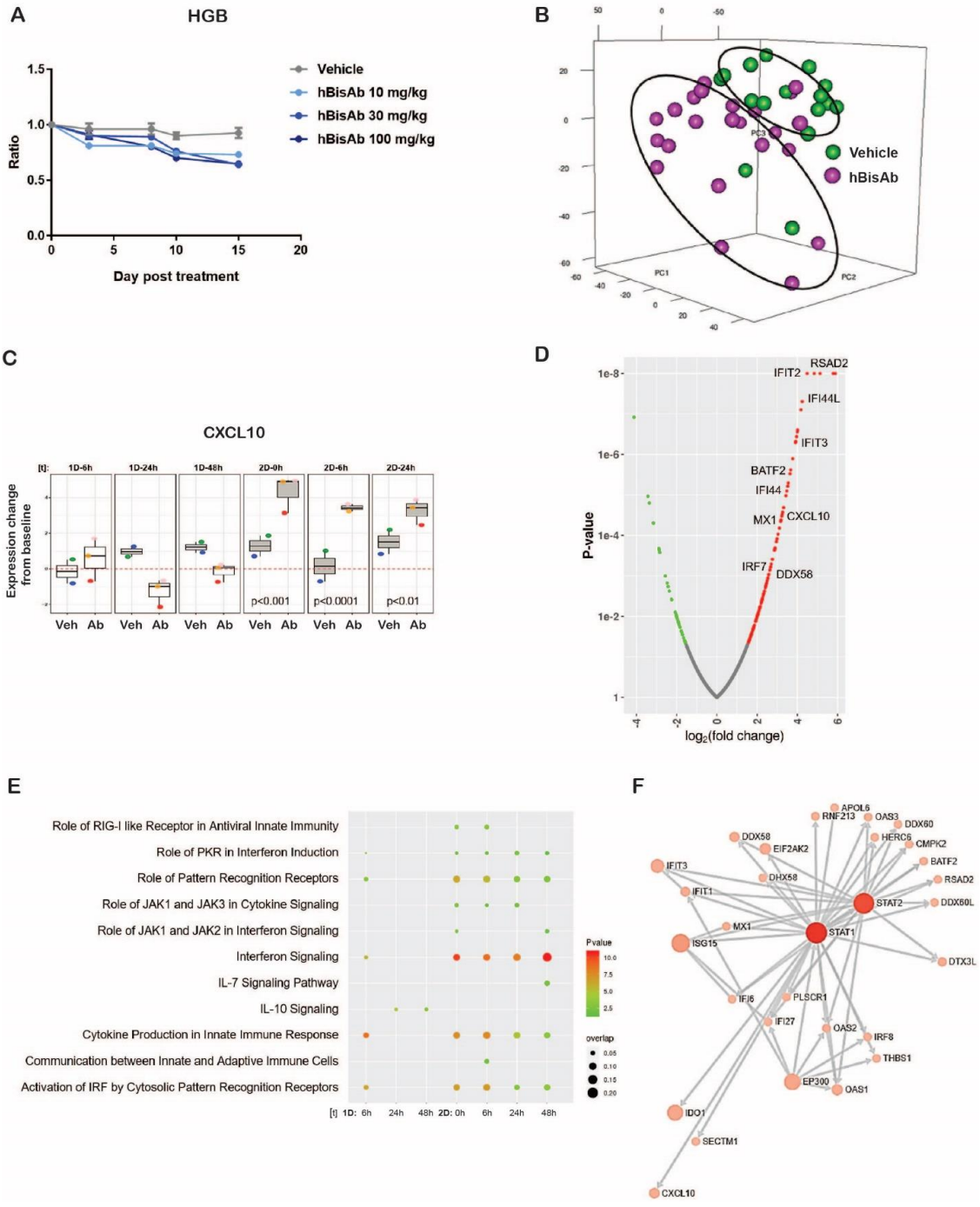


Supplementary Figure 5. scRNA-seq analysis on myeloid and DC clusters following mBisAb treatment (**A**) Single-cell expression of representative markers used to characterize intratumoral

monocyte and macrophage populations. (**B-D**) Relative average expression across treatment groups for selected genes that are differentially expressed among the indicated clusters in mBisAb- versus control-treated tumors. (**E-J**) Selected hits from GO pathway analysis performed on genes differentially expressed by the indicated clusters from mBisAb-treated tumors relative to controls. (**K**) Selected DEGs for DC clusters in mBisAb- versus control-treated tumors. Expression is displayed relative to all treatment groups and clusters.


A
Tcf7
Cxcr3
Ifit3
Ifit1
Ccl5
Tox
Pdcd1
Lag3
Cd244
Ifnar1
Cx3cr1
Ifng
Ccl3
Ccl4
Mki67
Expression Level
Cluster: 1 2 3 4 5 6
B
Control
mBisAb
9%
90%
27%
71%
Tcf1
Lag3
C
Tcf1+ Lag3- CD8 T cells
#/mg tumor
Control
mBisAb
D
GSE Analysis
Relative increase in Cluster 1 mBisAb vs Control
KAECH_NAIVE_VS_DAY8_EFF_CD8_TCELL_DN
GSE9650_NAIVE_VS_EFF_CD8_TCELL_DN
KAECH_NAIVE_VS_DAY15_EFF_CD8_TCELL_DN
KAECH_NAIVE_VS_MEMORY_CD8_TCELL_DN
GSE9650_NAIVE_VS_MEMORY_CD8_TCELL_DN
KAECH_DAY8_EFF_VS_DAY15_EFF_CD8_TCELL_UP
GSE9650_EFFECTOR_VS_MEMORY_CD8_TCELL_UP
GOLDRATH_NAIVE_VS_EFF_CD8_TCELL_DN
GSE41867_DAY6_EFFECTOR_VS_DAY30_EXHAUSTED_CD8_TCELL_LCMV_CLONE13_UP
-log10(q value)
E
Cd3e
Cd8a
1. Naive
2. Memory
3.Proliferating
4. Effector

Supplementary Figure 6. scRNA-seq analysis of CD8 T cell populations in the tumor and spleen following mBisAb treatment (**A**) Violin plots depicting normalized and log-transformed single-cell expression (logTPM, transcripts per million) of selected genes across CD8 T cell clusters. (**B,C**) Mice were treated as in Figure 6 and tumors were harvested at the same time points for flow cytometric analysis (n=5 mice/group). (**B**) Representative flow cytometry plots of $Tcf1^{hi}$ $Lag3^{lo}$ versus $Tcf1^{lo}$ $Lag3^{hi}$ populations among antigen-experienced ($CD44^{hi}CD62L^{lo}$) intratumoral CD8 T cells in control versus mBisAb-treated tumors. (**C**) Total numbers of $CD44^{hi}CD62L^{lo}$ $Tcf1^{hi}$ $Lag3^{lo}$ intratumoral CD8 T cells in control versus mBisAb-treated tumors. **P<0.01, Student's unpaired T-test. (**D**) Selected hits from gene set enrichment (GSE) analysis performed on genes differentially expressed by Cluster 1 intratumoral CD8 T cells from BisAb-treated relative to control-treated mice. (**E**) tSNE analysis of splenic $CD45^{+}$ cells from across all treatment conditions, with superimposed single-cell expression patterns of *Cd3e* and *Cd8a*, where each cell is colored according to the TPM value. Inset shows CD8 T cell clusters as determined by gene expression patterns in Figure 6G.



Supplementary Figure 7. Hemoglobin and gene expression analysis in NHP peripheral blood following hBisAb treatment (**A**) Cynomolgus monkeys received vehicle control and 10, 30, and

100 mg/kg of hBisAb on day 1 and 8 (n=1~3 monkey/group). The peripheral blood was collected at the indicated timepoint for hemoglobin (HGB) in response to the treatment. Ordinary one-way ANOVA with Dunnett's multiple comparisons test. (**B**) Principal-component analysis of RNA-seq samples from cyno peripheral blood receiving vehicle control or 100 mg/kg of hBisAb across all timepoints listed in Figure 7E. (**C**) *Cxcl10* gene expression change from baseline (0hr) comparing hBisAb- and vehicle-treated group across all timepoints (Student's unpaired T-test). (**D**) Volcan plot showing DEGs between hBisAb- and vehicle-treated group on 6h post-second dose on day 8. (**E**) IPA analysis of the DEGs from hBisAb-treated compared to vehicle group across all timepoints. (**F**) Gene correlation network analysis based on the interaction of top 100 up-regulated DEGs in hBisAb compared to vehicle group (fold change >2 and p-value > 0.05).

Supplementary Materials and Methods

Kinetic analysis

The analysis of affinities and kinetic binding constants of interactions between hBisAb and human, cynomolgus monkey, and mouse CD47 was performed by SPR using a Biacore T200 (Cytiva). hBisAb was immobilized on a surface via anti-human Fc antibody coupled to a CM4 chip and recombinant CD47 antigens were run over the surface as analytes. For PD-L1, the experiment was performed using neutravidin capture of biotinylated hBisAb and a single cycle kinetics methodology to capture the high-affinity interactions between hBisAb and PD-L1. The blocking activity of the interaction of SIRPα/CD47 and PD-1/PD-L1 was assessed in a biolayer interferometry binding assay (BLI), using an Octet HTX BLI instrument (Sartorius)

Production of αCD47/PD-L1 bispecific antibodies

For human and mouse bispecific antibodies, DNA sequences encoding αPD-L1 heavy chain, αCD47 heavy chain, and common lambda light chain were cloned into three separate mammalian expression vectors under the control of CMV-1 promoter using standard molecular biology techniques. For hBisAb, heterodimerization was achieved using 'knobs-into-holes' approach with αPD-L1 heavy chain harboring 'knob' mutations (T366W, Y349C) and αCD47 'hole' mutations (T366S, L368A, Y407V, S354C) in the human IgG1 background. For mBisAb, bispecific modality was achieved using 'R' mutations (T370K, K409R) in αPD-L1 heavy chain, and 'E' mutations (F405L, R411T) in αCD47 heavy chain in the mouse IgG2a background. In both cases 6xHis tag was added at the C-terminus of the αPD-L1 heavy chain to facilitate purification and separation of homodimeric monoclonal antibodies from desired heterodimeric bispecific antibody species. Expi293 cells were transfected transiently using 25 kDa PEIs (Polysciences) and all antibodies were expressed for 96 hours at 37°C.

Human bispecific antibodies were expressed after co-transfection of three chains (αPD-L1, αCD47 and common lambda light chain). Cells were harvested by centrifugation and resulting culture media was applied to ProteinA MabSelectSure column (Cytiva), purified according to manufacturer's protocol, and eluted proteins were dialyzed into PBS. Next, cation exchange column Source 15S (Cytiva) was used to separate homodimeric and heterodimeric species in 30 mM MES pH 6.0 buffer using gradients of 1 M NaCl (step 5-13% and linear 13-25%). Fractions containing hBisAb were dialyzed into PBS with 0.01% Tween-20 and frozen in aliquots at –80°C.

Mouse bispecific antibodies were purified from two separate monoclonal antibodies, followed by *in vitro* redox reaction to promote heterodimerization of the heavy chains. The αCD47 and αPD-L1 monoclonal antibodies were transfected as described above and culture media was purified using ProteinA MabSelectSure column (Cytiva) according to manufacturer's protocol, followed by desalting of eluted proteins into PBS using HiPrep 26/10 column (Cytiva). Equal amounts (mg:mg) of αCD47 and αPD-L1 monoclonal antibodies were mixed in PBS supplemented with 1 mM EDTA and 75 mM 2-MEA, and the reaction was incubated at 31°C with gentle shaking (300 rpm) for 5 hours. The reaction mixture was then buffer exchanged into PBS via dialysis to remove 2-MEA and EDTA and allow for mBisAb formation. Resulting reaction was then applied to cation exchange MonoS HR 16/10 GL column (Cytiva) and separated in 25 mM MES pH 5.5 buffer using 1 M NaCl linear gradient (1-100%). Fractions containing mBisAb were dialyzed into PBS and frozen in aliquots at –80°C.

Cell lines

CT26, B16F10, NCI-H292, HT1080 cells were obtained from the ATCC and cultured according to manufacturer's protocol. The MC38 murine colon carcinoma cell line was a gift from the Ribas Lab (UCLA) and were cultured in RPMI supplemented with 10% HI FBS, 1% Pen-Strep. CHO-

hPD-L1 and PD-1 Effector cells were obtained from Promega and cultured according to manufacturer's protocol. CHO-mPDL1 cells were obtained from GenScript and cultured according to manufacturer's protocol. CHO-hCD47 cells obtained from BPS Bioscience were cultured according to manufacturer's protocol. CHO-hCD47 cells and CHO-mCD47 cells were generated internally at Pfizer by stably transfecting CHO cells with human or mouse CD47 in pcDNA3.1(+) vector (Thermo Fisher Scientific) via G418 Sulfate selection media were cultured for the initial passages using CD CHO Medium supplemented with 10% HI FBS, 1% Pen-Strep plus 500 µg/ml of G418 Sulfate. All cell lines were cultured in a humidified chamber at 37°C under 5% CO2 atmosphere.

Animals

All mice studies were conducted according to the NIH animal care guidelines and following protocols approved by the Institutional Animal Care and Use Committee from Pfizer, Inc. C57BL/6, Balb/c, and Batf3-/- mice were obtained from the Jackson Laboratory and were 6-10 weeks of age at time of tumor inoculation. Human FcgR mice were bred in-house at Pfizer and were 30-38 weeks of age at time of tumor inoculation. Mice were of mixed sexes. Mice within experiments were age and sex matched. The NHP study was conducted in an AAALAC accredited facility (Pfizer, Groton, CT), in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare. The procedures used in this study have been reviewed and approved by the Institutional Animal Care and Use Committee. Cynomolgus monkeys (> 2.5 yrs old, body weight range of 3.4-6.6 Kg) of Mauritius origin were sourced through Covance Primates, screened and randomly assigned to study treatment groups.

Purification of human monocytes from PBMCs and differentiation to macrophages

PBMCs were isolated from individual healthy donor buffy coats or leukopaks. Buffy coats from two healthy donors' whole blood were received from Stanford Blood Center. Twenty (25) mL of buffy coat was diluted with 75 mL PBS. Then 30 mL of diluted buffy coat was layered on top of 20 mL Ficoll-Paque PLUS (GE Healthcare) in SepMate50 PBMC Isolation Tubes and spun at 2,200 rpm for 20 minutes, at room temperature. The PBMC layer was transferred into a new 50 mL conical and washed with PBS. Samples were spun at 1,200 rpm for 5 minutes at room temperature. Following centrifugation, supernatant was removed, and cells were resuspended in 3 mL ACK Lysing Buffer and incubated at room temperature for 3 minutes. Thirty (30) mL of PBS was added to tubes and tubes were spun at 1,200 rpm for 5 minutes. Supernatant was removed and cells were resuspended in PBS containing 2% HI FBS for monocyte isolation. Leukopaks from healthy donors' whole blood were received from HemaCare. ACCUSPIN System-Histopaque1077 tube were brought to room temperature and centrifuged at 1,000 x g for 30 seconds at room temperature to assure that the liquid in the ACCUSPIN System-Histopaque1077 tube was below the frit, and then 15 to 30 mL of leukopak was poured into the upper chamber of each ACCUSPIN System-Histopaque1077 tube and spun at 1, 000 x g for 10 minutes at room temperature. After centrifugation, the plasma layer was aspirated within 0.5 cm of the opaque interface containing PBMCs. Then the PBMC layer was transferred into a new 50 mL conical tube and washed with PBS. Tubes were spun at 300 x g for 8 minutes. Following centrifugation, supernatant was removed, and cells were resuspended in 3 mL ACK Lysing Buffer and incubated at room temperature for 3 minutes. Thirty (30) mL of PBS was added to tubes and tubes were spun at 300 x g for 5 minutes. Supernatant was removed and cells were either resuspended in PBS containing 2% HI FBS for monocyte isolation or were cryopreserved in HI FBS with 10% DMSO

for future use. $CD14^+$ monocytes were enriched from PBMCs using a $CD14^+$ positive selection kit from Stem Cell Technologies according to manufacturer's protocol using the 'Big Easy' EasySep magnet. Enriched $CD14^+$ monocytes were cultured in cell culture media composed of RPMI 1640 supplemented with 10% HI FBS, 1% Pen-Strep at 1 x 10^6 monocytes/mL. The $CD14^+$ monocytes were differentiated into macrophages by the addition of 20 ng/mL of M-CSF for 6 to 7 days with additional media supplemented with 20 ng/mL M-CSF added 2 to 3 days post start of culture. On the day before the phagocytosis assay, media was aspirated and replaced with fresh cell culture media supplemented with 20 ng/mL of M-CSF and 10 ng/mL of IL-10 and cultured for overnight. Cells were grown in a humidified chamber at 37°C under 5% CO2 atmosphere.

Binding to human CD47 or human PD-L1 by flow cytometry

CHO-hCD47 or CHO-hPD-L1 cells were assayed to determine the cell-based binding human CD47 EC50 or human PD-L1 EC50 for test compounds by flow cytometry. Cells were collected using TrypLE Express Enzyme, pelleted, and resuspended with FACS Buffer (PBS + 2% HI FBS). 50,000 cells were plated per well in 96-well round bottom plate and incubated with titrating doses of test compounds for 30 minutes on ice. Cells were washed with FACS buffer and resuspended with 100 µL of a 1:100 dilution of APC-labeled anti-hIgG (Fcγ Fragment Specific) antibody per well and incubated in the dark on ice for 30 minutes. Cells were washed and resuspended in cold FACS Buffer for data acquisition using a BD Fortessa. Data analysis was performed using Flowjo software. EC50 was then determined by non-linear regression plot of *log(agonist) vs. response – variable slope (four parameters)* with bottom and top constraints set in GraphPad Prism software.

SIRPα-CD47 blocking assay

CHO-hCD47 cells from BPS Bioscience and CHO-mCD47 generated in house were assayed to determine the cell-based SIRPα blocking IC50 for test compounds by flow cytometry. Cells were

collected using TrypLE Express Enzyme, pelleted, and resuspended in FACS Buffer (PBS + 2% HI FBS). 50,000 cells were plated per well in 96-well round bottom plate and incubated 1ug/well of recombinant SIRP alpha/CD172a Fc chimera protein and titrating doses of test compounds for 30 minutes on ice. After incubation, cells were washed with FACS buffer and resuspended with 100 µL of a 1:100 dilution of APC anti-human SIRPα/β antibody or APC-anti-mouse SIRPα per well and incubated in the dark on ice for 30 minutes. Cells were washed twice with FACS buffer and resuspended in cold FACS Buffer for data acquisition using a BD Fortessa. The cell-based hSIRPα blocking graph shows the percentage of SIRPα bound on CHO-hCD47. The cell-based mSIRPα blocking graph shows the APC MFI on CHO-mCD47. The cell-based SIRPα blocking IC50 value was determined by non-linear regression plot of log(inhibitor) vs. response – variable slope (four parameters) in GraphPad Prism software using the APC MFI (Median Fluorescence Intensity). Data analysis was performed using Flowjo software.

Human PD-1-PD-L1 TCR NFAT bioassay

The PD-1/PD-L1 blockade TCR NFAT bioassay (Promega) was used for the *in vitro* blocking activity of test compounds on the PD-L1/PD-1 axis. Adherent CHO-PD-L1 cells were collected using TrypLE™ Select Enzyme. Cells were centrifuged at 230 × g for 10 minutes and resuspended in cell recovery medium (Ham's F-12 Nutrient Mix supplemented with 10% HI FBS) to a concentration of 3×10^5 viable cells/ml. 30,000 cells were plated per well in a white, flat-bottom 96-well assay plate and incubated overnight in at 37°C under 5% CO2 atmosphere. The next day PD-1 effector cells were prepared by pelleting the cells at 130–180 × g for 10 minutes at ambient temperature and resuspending in assay buffer (RPMI 1640 with L-glutamine supplement with 1% HI FBS) to a concentration of $1.25x10^6$ cells/mL. Cell recovery medium was removed from the white, flat-bottom 96-well assay plate containing pre-plated CHO PD-L1 cells. $5x10^4$ PD-1

Effector cells were plated per well and incubated with titrating doses of test compounds for 6-24hrs at 37°C under 5% CO2 atmosphere. After incubation, assay plates were removed from the incubator and equilibrated to ambient temp for 5-10 minutes. Bio-Glo Reagent was added to the inner 60 wells of the assay plates and incubated at ambient temperature for 5–30 minutes. Then luminescence was measured using a luminescence plate reader. Data analysis was performed using Excel and Prism. Fold induction was calculated using the following formula Calculate fold induction = RLU (induced)/RLU (no antibody control). The TCR EC50 was determined by non-linear regression plot of log(agonist) vs. response – variable slope (four parameters) with bottom and top constraints set in GraphPad Prism software.

Mouse PD-1/PD-L1 blocking assay

CHO-mPD-L1 cells from GenScript were assayed to determine the cell-based mPD-L blocking IC50 for test compounds by flow cytometry. Cells were collected using TrypLE Express Enzyme, pelleted, and resuspended in FACS Buffer (PBS + 2% HI FBS). 50,000 cells were plated per well in 96-well round bottom plate and incubated 0.6 ug/well of Recombinant Mouse PD-1 Fc Chimera Protein and titrating doses of test compounds for 30 minutes on ice. After incubation, cells were washed with FACS buffer and resuspended with 100 µL of a 1:200 dilution of APC-labeled anti-hIgG (Fcγ Fragment Specific) per well and incubated in the dark on ice for 30 minutes. Cells were washed twice with FACS buffer and resuspended in cold FACS Buffer for data acquisition using a BD Fortessa. The cell-based mPD-1 blocking graph shows the APC MFI on CHO-mPD-L1 cells as determined by non-linear regression plot of log(inhibitor) vs. response – variable slope (four parameters) in GraphPad Prism software using the APC MFI (Median Fluorescence Intensity). Data analysis was performed using Flowjo software.

***In vitro* binding selectivity assay**

To determine RBC versus tumor cell binding selectivity of test compounds, RBCs and CFSE labeled tumor cells were co-cultured at a 1:1 ratio with test compounds. Adherent HT1080 or MC38 cells were collected using TrypLE Express Enzyme and pelleted. Tumor cells were stained with 4ul CFSE stock solution for 10 minutes at 37°C under 5% CO2 atmosphere. CFSE labeled tumor cells were washed, pelleted, and resuspended at 2.5 x 10^6 cells/mL in FACS buffer. Human or murine RBCs were counted using a hemacytometer and cell density was calculated. The required number of RBCs were aliquoted and resuspended at 2.5 x 10^6 cells/mL in FACS buffer. Test compounds were diluted in FACS buffer (PBS containing 2% HI FBS) serial dilutions were prepared. Fifty thousand (50,000) CFSE labeled tumor cells and 50,000 RBCs were plated per well in 96-well round bottom plates and incubated with test compounds for 30 minutes on ice. Cells were washed with PBS and resuspended with 100 µL of a 1:200 dilution of AF647-labeled anti-hIgG (Fcγ Fragment Specific) or APC-labeled anti-mIgG (Fcγ Fragment Specific) antibody per well and incubated in the dark on ice for 30 minutes. Cells were washed with FACS buffer and analyzed on a Fortessa with FACS Diva software (BD Biosciences). CFSE was detected as FITC and AF647 was detected as APC on the Fortessa. Tumor cells were defined as FITC+ and RBCs were defined as FITC negative (FITC). Cells that stained positive for APC (APC+) were defined as antibody bound cells. The frequency of APC+ cells within the FITC+ population or FITC- population was used as the 'percentage of antibody-bound cells per cell type'. The frequencies were exported from Flowjo and analyzed in PRISM. The tumor and RBC binding EC_{50} values were determined by non-linear regression plot of log(agonist) vs. response – variable slope (four parameters*)* with bottom and top constraints set in GraphPad Prism software. Treatment concentrations plotted as agonist. Percentage of antibody-bound cells per cell type was plotted as

response. Bottom constraint equal to the 0% antibody bound. Top constraint equal to the 100% antibody bound.

Phagocytosis assay

To test antibody-induced phagocytosis of IFNg treated NCI-H292 cells by monocyte-derived macrophages, NCI-H292 cells were treated with 10 ng/mL of recombinant human IFNg overnight to increase PD-L1 expression. NCI-H292 cells were used as a representative tumor cell line. On the day of the assay, adherent NCI-H292 cells and macrophages were treated with TrypLE. The cells were collected in 50 mL conical tubes and pelleted by centrifugation at 1,200 rpm for 5 min, and then supernatant aspirated and resuspended in 1 mL of PBS for CSFE and CTV staining. Stock solutions of CFSE and CTV were prepared by dissolving the contents of one vial of CFSE or CTV (lyophilized powder) with 50 µL of DMSO prior to use. To stain NCI-H292 cells, 2 µL of CFSE stock solution was added to 1 mL of cells. To stain macrophages, 2 to 4 µL of CTV stock solution was added to 1 mL of cells. The cells were incubated for 10 minutes at 37°C under 5% CO2 atmosphere. After 10 minutes of staining cells, 15 mL of PBS was added to tube and cells were centrifuged at 1,200 rpm for 5 minutes. CFSE stained NCI-H292 cells were resuspended to in assay media (RPMI 1640 supplemented with 10% HI FBS and 1 % Penstrep). CTV stained macrophages were resuspended in assay media. Test compounds were diluted in assay media and 7-point 5-fold serial dilutions were prepared. Twenty-five thousand (25,000) CFSE labeled, IFNg treated NCI-H292 cells and 50,000 CTV labeled macrophages were plated per well in 96-well round bottom plates and incubated with test compounds (dose range 0.0128 nM to 200 nM) for 2 hours at 37°C under 5% CO2 atmosphere. After incubation, co-cultures were washed with 100 µL PBS and spun down at 1,200 rpm for 5 minutes. Supernatant was removed and each well resuspended in 120 µL of FACS Buffer (PBS supplemented with 2 % HI FBS). Then each well

was acquired on the Fortessa with FACS Diva software. CFSE was detected as FITC and CTV was detected as BV421 on the Fortessa. For each well, 5,000 FITC+ events were collected. Data analysis was performed using FlowJo software. Samples were gated to exclude doublets. Tumor cells were defined as FITC+ and macrophages were defined as BV421+. Cells that were FITC+BV421+ were defined as tumor cells phagocytosed by macrophages. Cells that were FITC+BV421- were defined as non-phagocytosed tumor cells. The number of events for phagocytosed and non-phagocytosed tumor cells were exported from FlowJo into Excel. In Excel, the percent phagocytosis of total tumor cells was calculated using the following formula:

(No. of phagocytosed tumor cells) / (No. of phagocytosed tumor cells + No. of non-phagocytosed tumor cells) * 100 = Phagocytosis % of Total Tumor

Percent phagocytosis of total tumor cells was plotted in GraphPad Prism Software.

MLR assay

LPS matured DCs were prepared. Isolated human PBMC were resuspended to $1x10^6$ cell/ml with Serum-Free RPMI Medium 1640 (Gibco A10491-01) and incubated at 37 degree incubator for 3 hrs. Then supernatant was discarded, RPMI 1640 Medium + 10 %FBS (Biological Industries 04-002-1A) with 250U/ml IL-4 (Peprotech 200-04) and 500 U/ml GM-CSF (Peprotech 300-03) was added and cell were incubated in 37 degree incubator for 5-7 days. Then the DCs were matured with 1μg/ml LPS (sigma L6529) in RPMI 1640 Medium + 10 %FBS for about 24 hrs. CD4+ T cells were isolated from a freshly isolated human PBMC (different donor than DCs) according to the kit (Easysep human CD4+ T cell isolation, Stemcell#17952). 200,000 purified CD4+ T cells and 50,000 matured DC were added to 96 well plate with test compounds. Samples were incubated at 37 degree for 72 hours for IL-2 detection and 120 hours for IFN-gamma detection. Supernatants were harvested at 72 and 120 hours and frozen at -20 degree for ELISA assay. IL2 was detected

in supernatant by ELISA kit (R&D#DY202) according to manufacturer protocol. IFNg was detected in supernatant by ELISA kit (R&D#DY285B) according to manufacturer protocol.

Preparation of single cell suspension from mouse tissues

For characterization of splenic and tumor infiltrating T cell populations, spleens were mechanically dissociated by processing over a 40uM nylon mesh filter before treatment with HybriMAX Red Blood Cell Lysing Buffer (Sigma Aldrich) at room temperature for 5 minutes, and tumors were dissociated and processed to a single suspension using the Mouse Tumor Dissociation Kit (Miltenyi Biotec) according to manufacturer's instructions.

For assessment of *in vivo* binding selectivity, tumors were mechanically dissociated by using the gentleMACS OCTO Dissociator and then filtered over 70uM nylon mesh filter. Blood samples were collected in heparin diluted 1:20 in PBS, then samples were washed with FACS buffer (PBS + 2% Heat Inactivated FBS)

For characterization of splenic and tumor infiltrating T cell populations by CyTOF, spleens were treated for 25 minutes at 37 degrees with 25 ug Liberase TL (Roche) then mechanically dissociated by processing over a 70uM nylon mesh filter before treatment with ACK Lysis Buffer (Gibco) at room temperature for 3 minutes. Tumor samples were dissociated and processed to a single suspension using the Mouse Tumor Dissociation Kit (Miltenyi Biotec) according to manufacturer's instructions except for 10% less than recommended amount of the R enzyme was used per sample. Single cell suspension of tumor samples were then enriched for CD45+ cells using the Miltenyi mouse CD45(TIL) Microbeads Kit (Miltenyi BioTec) according to manufacturer's instructions for the LS columns.

For characterization of splenic and tumor infiltrating T cell populations by scRNA-seq, spleens were processed to a single cell suspension using the same method used for processing spleens in the CyTOF study. Tumors were mechanically dissociated according to the protocol used for *in vivo* binding selectivity. Single cell suspension of tumor samples were then enriched for CD45+ cells using the Miltenyi CD45 MicroBeads Kit (Miltenyi BioTec) according to manufacturer's instructions.

Mass Cytometry (CyTOF)

Mass-tagged Antibodies

A list of all mass cytometry antibodies and their labelled isotopes are summarized in Supplementary Table 9. Pre-conjugated metal-tagged antibodies were purchased from Fluidigm when available. For all other antibodies, purified formulations were purchased from the companies indicated in Supplementary Table 9, and conjugated using the Maxpar® X8 Antibody Labelling Kit (Fluidigm) according to the manufacturer's protocol.

Mass Cytometry Surface and Intra-cellular Straining

Three million CD45+ TIL enriched cells from each sample were washed with PBS (minus Ca^{2+} and Mg^{2+}) (Gibco) to remove soluble proteins, and labelled with Cell-ID™ Cisplatin-198Pt (Fluidigm) for 5 minutes to measure viability. Cells were then washed with Maxpar® Cell Staining Buffer (SB) (Fluidigm) and normalized to 100 µL of residual volume before surface marker staining for 40 mins. at room temperature. Cells were blocked with 5 µL of purified anti-mouse CD16/32 (clone 93) antibody (Biolegend) for 5 mins. prior to staining. Cells were then washed with SB before they were fixed and permeabilized using the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (Invitrogen™, Waltham, USA). Cells were normalized to 100 µL

residual permeabilization buffer, before intra-cellular marker staining for 40 mins. at room temperature. Cells were washed once more with permeabilization buffer, and then washed with SB before mass-tagged barcoding. After barcoding and pooling all samples into batches, each batch was diluted to $2x10^6$ cells/mL of DNA-Ir solution and stored at 4°C for up to 10 days prior to acquisition on the Helios™ (Fluidigm) mass cytometer. DNA-Ir consists of 2.0% (w:v) EM Grade Paraformaldehyde (Catalog#15710, Electron Microscopy Sciences) in PBS, with 125 nM Cell-ID™ Intercalator-Ir (Fluidigm).

Mass-tagged Cellular Barcoding

All samples were labelled per manufacturer's protocol, with metal-tagged cellular barcodes using the Cell-ID™ 20-Plex Pd Barcoding Kit (Fluidigm). Barcoded samples were pooled into batches of up to 20 samples, so that no two samples shared the same hexameric barcode within each batch. Each individual sample within each batch was reidentified by manual boolean gating of palladium (Pd) isotopes ^{102}Pd, ^{104}Pd, ^{105}Pd, ^{106}Pd, ^{108}Pd, and ^{110}Pd, and then exported as its own FCS file.

Acquisition & Bead Standard Data Normalization

An aliquot from each batch, was washed twice with SB and then twice with ≥18.2 MΩ.cm Milli-Q® ultrapure water (MilliporeSigma). Aliquots were stored as a pellet in residual volume for no longer than 16 hours at 4°C. Right before acquiring, each aliquot was filtered through a 35 μm nylon mesh (Catalog# 352235, Falcon), and resuspended to $1.0x10^6$ cells/mL of diluted (1:4 parts) EQ™ Four Element Calibration Beads (Fluidigm). Instrument tuning was performed once per day of operation according to manufacturer's protocol, unless a part was replaced in which cased tuning was run again. An event rate of 500 per second or less was maintained for all aliquots. Using the PSI injection system, a sample line pressure of <20 PSI was maintained by intermittent cleaning using 2.0% (v:v) nitric acid and manual backflushing, or by replacing parts that had

impeded flow due to sample clogging. All recorded FCS data was randomized, using the uniform negative distribution method, and was bead-normalized, using median bead intensity at 100 second intervals, with the instrument's CyTOF® Software (v6.0).

Individual sample FCS data were manually gated using OMIQ SaaS (Omiq Inc.) to exclude normalization beads, cell debris, dead cells, technical artifacts, and doublets for the identification of live CD45+ cells prior to downstream analyses.

Flow cytometry

For characterization of splenic and tumor infiltrating T cell populations, single cell suspensions from tumor and spleen were stained with the following surface antibodies in a 1:1 dilution of Staining Buffer (Biolegend) and BD Horizon Brilliant Stain Buffer (BD Biosciences) for 10 minutes on ice: anti-CD45, NK1.1, CD19, CD4, CD8a, CD3, TCRb, CD25, CD44, CD62L, and Lag3. Samples were then stained in LIVE/DEAD Fixable Blue Cell Stain Kit (Thermo Fisher Scientific) at 1:1000 in PBS on ice for 10 minutes. Following fixation for 30 minutes on ice using the FoxP3 Transcription Factor Staining Buffer Set (Thermo Fisher Scientific), cells were incubated with intracellular antibodies (anti-Ki67 and Tcf1) in permeabilization buffer for 8 hours at 4C. Samples were washed twice prior to acquisition on an Aurora (Cytek). Additional details regarding the specific antibodies used for flow cytometric analysis are provided in Supplementary Table 9.

For assessment of *in vivo* binding selectivity, blood samples and single cell suspension from tumor and spleen were stained in mouse Fc block (BD) at 1:25 and LIVE/DEAD Fixable Blue Cell Stain Kit (Thermo Fisher Scientific) at 1:100 in FACS buffer at room temperature for 10 minutes. Then samples were stained with the following surface antibodies in a 1:1 dilution of FACS buffer and BD Horizon Brilliant Stain Buffer (BD Biosciences) for 1 hour on ice: anti-CD45, anti-B220, anti-

Thy1.2, anti-CD4, anti-CD8, anti-NKp46, anti-CD11b, anti-CD11c, anti-F4/80, anti-Ly6C, anti-Ly6G, anti-IL4R and anti-human IgG (Jackson ImmunoResearch). Samples were washed twice prior to acquisition on an Fortessa (BD).

Single-cell RNA-seq and data processing

Tumors were harvested and viable CD45+ cells were FACS sorted from control, αmCD47, αmPD-L1, αmCD47 and αmPD-L1 combination, and mBisAb-treated mice (n = 4~5/group). Single-cell emulsions were obtained using the 10X Genomics Controller and the v2 library and Gel Bead kit (10x Genomics). RNA-sequencing libraries were prepared as instructed by the 10x 3' v2 kit protocol. Resulting libraries were sequenced on an Illumina NextSeq using a NextSeq 500/550 v2.5 High Output Kit. Cell Ranger 3.0.2 (https://support.10xgenomics.com/) was used to process single-cell sequencing data and generate the matrix data containing gene counts for each cell per sample. Briefly, raw base call files from Illumina sequencers were first demultiplexed into FASTQ files with the "cellranger mkfastq" pipeline. Then, the "cellranger count" was used to align FASTQs files to the mouse reference genome (mm10) followed by reads filtering, barcode counting, and UMI counting. Finally, the gene expression matrixes of all samples were imported into Seurat v3(3.2.3). The following filtering steps were carried out to exclude low-quality cells: (a) cells with fewer than 200 detected genes and/or (b) cells with greater than 10% mitochondrial reads were discarded. As a result, a total of 13667 cells (2269 from control, 2319 from αmCD47-treated, 2915 from αmPD-L1-treated, 2239 from αmCD47 and αmPD-L1 combination-treated, 3925 from mBisAb-treated) with 17556 informative genes were included in the downstream analyses.

Clustering of single cells, trajectory analysis, and definition of cell states

The gene expression data were log-normalized and scaled with default parameters. The top 2,000 most variable genes identified by Seurat function "FindVariableFeatures" were used for the principal component analysis (PCA). The first 40 principal components (PCs) selected based on the ElbowPlot were used for clustering analyses. Cell clusters were identified using FindClusters functions implemented in Seurat with default parameters and resolution parameter as 0.9. The t-SNE and UMAP were used to visualize the clustering results with default parameters. Myeloid cells and lymphocytes were further separated into different subtypes based on the same procedures. Machine learning algorithm Random Forest and our in-house reference databases were used to annotate the cell type of large cell populations. The cell types of clusters and subclusters were further confirmed and annotated by comparing the specifically expressed genes identified by the Seurat "FindAllMarkers" function with the known cell markers reported in the literature. Pathway scores were calculated by Suerat function "AddModuleScore" which calculate the average signature gene expression of each cluster subtracted by the aggregated expression of control gene set.

The myeloid cell subpopulations of interest were selected for single-cell trajectory analysis. Using Monocle (v2.0), cells were ordered according to their inferred pseudotime by following the steps described on Monocle documentation (http://cole-trapnell-lab.github.io/monocle-release/docs/). Only the top 100 differentially expressed genes among myeloid clusters were used for dimensionality reduction and trajectory reconstruction. The reduce Dimension function with DDRTree as the reduction method was applied to the top principal components (PCs) and projected the cells onto two dimensions. After the dimension was reduced, the "orderCells" function was used to order cells and the plot_cell_trajectory function was used to visualize the trajectory in two-dimensional spaces.

Gene expression analysis in mice

Total RNA (50–100 ng) was used in the nCounter assay (NanoString Technologies) using mouse-specific nCounter PanCancer Immune Profiling and PanCancer Pathways Panels supplemented with custom-made 30-plex code set or human-specific nCounter PanCancer Immune Profiling Panel following the manufacturer's recommendations. Samples were prepared using an nCounter Prep Station, and code set/RNA complexes were immobilized on nCounter cartridges for data collection; data were collected on a NanoString Digital Analyzer. nCounter RNA count data were normalized using the geometric mean of the positive controls and HKGs. Extensive quality control analysis was applied to raw and normalized data to identify batch effects and issues with control probes and the samples. A LLOD threshold was set for the counts on the basis of the maximum count of the background controls (negative). Genes with normalized counts less than LLOD were flagged as low-expressing genes. The curated data were subjected to statistical analysis using ANOVA tests followed by pathway analysis using IPA and GSEA.

Gene expression analysis in NHP

RNAseq sequences were aligned to Cyno genome (Macaca_ fascicularis_5.0.93 assembly and gtf) and gene expression quantification was performed by RSEM software (v1.3.0) using default parameters to calculate log2 TPM values. Differential gene expression analysis was performed using Limma (v3.38.3) with differentially expressed (DE) genes defined as those with adjusted p value <0.05 and fold-change > 1. Pathway analysis was performed on DE genes using "enricher" function in "clusterProfiler" R package (v3.14.3) and IPA database. Log2 transformed gene expression estimates (tag count per million, TPM) were used for the heatmaps. Relative expression to the baseline (0 hr) was plotted in the boxplot when comparing across different treatments and timepoints. LM22 (22 immune cell types) scores from all samples were calculated using average

expression of the genes in each signature. LM22 is a specific gene signature containing 547 genes that distinguish 22 immune cell subtypes downloaded from the CIBERSORT web portal (http://cibersort.stanford.edu/).