Colocalized inhibition of TGF-β and PD-L1

SUPPLEMENTARY MATERIALS

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SUPPLEMENTARY METHODS

Cell Lines

HEK293 parental cells were obtained from ATCC and cultured in DMEM (high glucose) medium with 1% GlutaMax and 1% MEM sodium Pyruvate. HEK293-PD-L1 cells were generated by overexpression of PD-L1 in HEK293 cells and cultured in RPMI1640 medium with 100 µg/mL Hygromycin B. 4T1 parental cells were obtained from ATCC and cultured in RPMI1640. 4T1-pSmad Luc cells were generated by overexpression of pSmad Luciferase cassette in 4T1 cells and cultured with 3 µg/mL Puromycin. All HEK293 and 4T1 cell lines were supplemented with 10% fetal bovine serum (FBS), 1% Penicillin, and 1% Streptomycin.

Detroit 562 cells were purchased from ATCC and cultured in MEM medium with 10% FBS and supplemented with 1% Penicillin, 1x MEM Sodium Pyruvate, and 1x MEM Non-Essential Amino Acids. Detroit 562 cells overexpressing PD-L1 were cultured in the same medium as the parental cells plus 1 µg/mL Puromycin. PBMCs (donor IDs: KP54530, KP55758, KP50665, KP56950, KP57059, KP57134, KP57166) were obtained from RBC (Research Blood Components, LLC).

The MC38 murine colon carcinoma cell line was a gift from the Scripps Research Institute, and cells were cultured in DMEM supplemented with 10% FBS. All cell lines were tested and verified to be free of adventitious viruses and mycoplasma.

Mouse Models

C57BL/6 and μMt$^-$ mice were obtained from Charles River Laboratories and Jackson Laboratory. All mice used in the studies were 8-12 weeks of age. All procedures were performed in accordance with institutional protocols approved by the Institutional Animal Care and Use Committees of the EMD Serono Research & Development Institute, Inc., (Billerica, MA); an affiliate of Merck KGaA (Darmstadt, Germany). StudyLog software was used to manage and organize in vivo experiments, randomize mice into treatment groups, and collect tumor volume data.

Murine Tumor Model and Treatment

All treatments were started on Day 0 when the mice were randomized into treatment groups.

For survival and complete regression analyses, MC38 cells (0.5×10^6) were inoculated intramuscularly (im) into μMt$^-$ mice (Day -7). Mice (n=14 per group) were intravenously (iv) injected with isotype control (400 μg, anti–PD-L1(mut)), the combination of anti–PD-L1 (133 μg) and TGF-β trap (164 μg; anti–PD-L1(mut)/TGF-β trap) [labeled as low dose], BA (164 μg) [labeled as low dose], the combination of anti–PD-L1 (400 μg) and TGF-β trap (492 μg) [labeled as high dose], or BA (492 μg) [labeled as high dose] on Days 0, 3, and 6.

For immunophenotyping of TILs, MC38 cells (1×10^6) were inoculated subcutaneously (sc) in 0.1 mL PBS into the flank of C57BL/6 mice (Day -14). Mice (n=8 per group) were intravenously (iv) injected with isotype control (400 μg), mouse IgG1 isotype control (200 μg), TGF-β trap (492 or 164 μg), fresolimumab (200 μg, anti–TGF-β), anti–PD-L1 (400 μg), BA (492 μg), the combination of anti–PD-L1 and fresolimumab, or the combination of anti–PD-L1 and TGF-β trap on Days 0, 1, and 2. Tumors were harvested and weighed on Day 6.

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For RNAseq and scRNAseq, MC38 cells (1 x 10^6) were inoculated subcutaneously (sc) in 0.1 mL PBS into the flank of C57BL/6 mice (Day -15). Mice (n=15 per group for bulk RNAseq, n=3 per group for scRNAseq) were iv injected with isotype control (400 μg), TGF-β trap (492 μg), anti–PD-L1 (400 μg), BA (492 μg), or the combination of anti–PD-L1 and TGF-β trap on Days 0, 1, and 2. Mice were sacrificed at Day 6 and tumor tissue was harvested and snap frozen (for bulk RNAseq) or mechanically dissociated into single cell suspension, enriched for viable cells via flow cytometry, then cryopreserved (for scRNAseq).

Biophysical Characterization

For biophysical characterization, the recombinant human TGF-β 1, 2 and 3 were obtained from R&D Systems (Cat#'s 7754-BH/CF, 302-B2/CF, 8420-B3/CF). PD-L1, BA, BA one-leg, fresolimumab, fresolimumab Fab, and fresolimumab one-arm were all synthesized in-house using mammalian expression. BA one-leg is the monovalent variant of BA that lacks one of the two TGF-βRII ECD fusion domains. Fresolimumab Fab is the Fab of reference antibody fresolimumab that exhibits only monovalent binding to TGF-β isoforms. Fresolimumab one-arm is the Strand Exchange Engineered Domain (SEED)-based monovalent variant of reference antibody fresolimumab that lacks one Fab portion. A Biacore T200 instrument (GE Healthcare) was used to measure biochemical binding by SPR. TGF-β1, 2, or β3 were immobilized by amine coupling on a CM5 chip to target density of 100 RU. Analyte proteins were bound at 40, 20, 10, 5, 2.5, 1.25, 0.63, 0.31 and 0 nM. Low immobilization density was used to decrease avidity effects in binding between the immobilized ligand and analyte. Association phase was for 600 seconds at 10 μl/mL and dissociation phase was for 900 seconds at 10 μl/minute. Kinetic rate constants were determined from fits of association and dissociation phases, and K_D was derived as the ratio of these constants (K_D = kd/ka).

For ITC, the Microcal (Malvern) VP-ITC Microcalorimeter was used. All proteins were transferred into the titration buffer (PBS) by dialysis. In order to remove any denatured protein contamination, the samples were centrifuged at 100000xg for 1 hour. Supernatants were collected and subjected to protein concentration determinations via optical density measurements of tryptophan absorbance at 280 nm in a Nanodrop device. TGF-β1 was loaded into the sample cell at concentrations of 3-5 µM of the homodimer. The ITC syringe was loaded with the respective interacting protein diluted to a final concentration of 50-80 µM in PBS. After assembly of the injector, the titration was conducted by additions of 12 µl aliquots from the syringe to the sample cell under continues stirring with 300 rpm. All titrations were done at 25°C and at least with two independent repetitions. For all fits, a binding model of one binding site was assumed. The titration of TGF-β1 dimer with BA revealed an exothermic binding signal that saturated in a steep symmetrical binding isotherm. Thus, in these cases it was only possible to estimate the upper bound for K_D value of $K_D \leq 2$ nM.

DLS was measured by the Prometheus Panta instrument from Nanotemper Technologies (Munich, Germany). Proteins or mixtures thereof were prepared as for ITC measurements, diluted to final concentrations of 0.1-1 mg/mL in PBS and filled into capillary cells for measurements.

For MST, the Monolith NT.115 Pico (Nanotemper Technologies) was used. TGF-β1 fluorescent labeling was accomplished at a protein concentration of 1.7-10 µM and a dye (Fluorescent dye 647 nm NHS) to protein of 3.5:1. In the MST assay, labeled TGF-β1 dimer was used at concentrations of 400 pM and 1 nM, respectively. Binding affinities were derived from dose response curves that were generally analyzed (Nanotemper software MO.Affinity Analysis) with a four-parameter fit to determine the K_D value. In cases of apparent binding affinities at or below the concentration of labelled TGF-β1, a Hill fit was used.

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Mass photometry measurements were done with a Refeyn OneMP instrument (Refeyn Ltd.; Oxford, UK). In brief, purified proteins or mixtures thereof were prediluted in PBS in a low-protein-binding test tube to a desired concentration in the range of 0.8-150 nM and finally diluted (1:10) into a drop of PBS-buffer that was positioned on a glass coverslip in the optical path of the instrument. Immediately after the dilution, the optical contrast change events were recorded over a period of one minute. Data were analyzed with the Refeyn Discovery MP software. For each molecular species, the mass histogram was analyzed with a Gaussian fit and the center mass was considered as the apparent molecular weight. The absolute mass values were obtained by referencing to a molecular weight standard (NativeMark Unstained Protein Standard-5, Life Technologies).

For negative stain electron microscopy, copper grids (300 mesh) were coated with ultrathin carbon film and glow discharged. Proteins were diluted to 10 µg/mL in PBS buffer and deposited on the grids and incubated for 40 seconds at room temperature. Excess liquid was removed, and the grids were washed in four droplets of double-distilled water and one droplet of 2% uranyl acetate (pH 4.3). The surface was negatively stained by incubation with an additional droplet of 2% uranyl acetate (pH 4.3) for 45 seconds at room temperature. Finally, excess liquid was blotted off, and the grids were kept at room temperature to air-dry. For each sample, 100 micrographs were collected on Tecnai G2 Spirit (FEI) operated at 80kV and equipped with a side mounted EMSIS MORADA camera. Micrographs were recorded using DigitalMicrograph (Gatan) at a nominal magnification of 105,000 x, resulting in a pixel size of 4.4Å. To maximize the contrast, a defocus range of 200 nm to 1µm and an exposure time of 1 second were applied. Image processing was performed using the EMAN2 image processing suite[1]. 5364 and 5197 particles were manually picked for BA and BA/TGF-β1-dimer complex, respectively. Picked particles were subjected to

multivariate statistical analysis yielding 50 classes. The best class averages were then selected based on the quality of the signal-to-noise ratio for comparison.

Proliferation Assay using Detroit Cells at Low-Cell Density

Detroit cells were plated at 80 cells/well in a 96-well plate and treated with one dose of BA, TGF-β trap, or fresolimumab at various concentrations, including 3x and 5x serial dilutions starting at 2,000 ng/mL and ending after 8 concentrations. After 2 weeks, a CellTiter-Glo 2.0 (Promega) assay was used to measure cell numbers, following the manufacturer's protocol. To neutralize the binding of BA to PD-L1, cells were pre-incubated with 40 μg/mL anti–PD-L1 antibody for 30 minutes before adding BA.

Two-way MLR Assay

Frozen PBMCs from two different donors were co-cultured at a 1:1 ratio for 3 days and treated with isotype control, BA, anti–PD-L1, TGF-β trap, or the combination of anti–PD-L1 and TGF-β trap, at 10 μg/mL. Results from 6 assays with 6 different donor pairs (KP54530+KP55758, KP50665+KP55758, KP50665+KP54530, KP56950+KP57059, KP57059+KP57134, KP57059+KP57166) were plotted together as fold changes over isotype control hIgG1 (set to 1). In a second study, frozen PBMCs from two different donors were co-cultured at a 1:1 ratio for 3 days and treated with BA and the combination of anti–PD-L1 and TGF-β trap at 10, 100, 1000 and 10000 ng/mL. Results from 4 assays (KP57059+KP57134, KP57059+KP57166, KP57059+KP56950, KP50665+KP55758) with 4 different donor pairs were plotted together as fold changes over BA at 10 ng/mL (set to 1).

Flow cytometry analysis

For the immunophenotyping of TILs, a single cell suspension was made using mechanical dissociation. Briefly, tumors were cut into 1-2 mm pieces and gently crushed between two frosted

glass slides. Cold staining buffer (2% FBS in PBS) was added, and the suspension was passed through a 70 μm strainer. After washing, the cells were resuspended in red blood cell (RBC) lysis buffer, and viable cells were analyzed by flow cytometry. Tumor weight was used to determine the absolute tumor cell counts.

To prepare cells for flow cytometry staining, Fc blocking antibody (anti–mu CD16/CD32, BD Pharmingen, 553142) was added to each sample for 15 minutes on ice. Cells were then incubated with fluorescent-conjugated primary antibodies. The MHC Pentamer P15E, which is a MC38 tumor antigen-specific, was used to identify a subpopulation of $CD8^+$ TILs specific to antitumor immune activity. For P15E staining, MC38 tumor cells were incubated with R-PE labeled Pro5 MHC Pentamer (Proimmune, F828-2B) prior to primary antibody staining.

For the analysis of MC38 TILs, the fluorophore-conjugated to Ki-67 (MOPC-21) was purchased from BD Biosciences and (P15E) H-2Kb KSPWFTTL was purchased from Proimmune. Antibodies to CD3 (17A2), CD8a (53-6.7), CD4 (RM4-5), CD44 (IM7), and CD45 (30-F11) were purchased from Biolegend. Viability Dye eFluor 455 (UV) and the antibody for CD62L (MEL-14) was purchased from eBiosciences.

After primary antibody staining, cells were washed and incubated with fixable viability dye (Invitrogen, 65-0868-18). Cells were then fixed and permeabilized with cytofix/cytoperm buffer (BD Bioscience, 554722) then washed in perm/wash buffer (BD Bioscience, 554723), blocked with Fc blocking antibody for 15 minutes, and incubated with the appropriate antibody solution in perm/wash buffer and incubated overnight at 4°C. Cells were washed and resuspended in FACS buffer prior to flow cytometry analysis.

For intracellular staining, single cell suspensions were stimulated with 50 ng/mL PMA (Sigma, Cat #: 356150010) plus 500 ng/mL ionomycin (Invitrogen, Cat #: 124222) in the presence

of 0.67 µl/mL GolgiStop (BD Bioscienses Cat #: 554724) for 4-6 hours at 37ºC in a humidified 5% CO_2 atmosphere. The cells were then prepared for flow cytometry staining as described above. Cells were analyzed on a BD LSR II flow cytometer according to the manufacturer's instructions. Flow cytometry analysis was performed using Flowjo software.

Bulk RNAseq Analysis

To perform transcriptome profiling, total RNA was extracted from the frozen tissue and whole transcriptome RNAseq was performed by MedGenome Inc. Raw sequencing data in FASTQ format was transferred to EMD Serono Bioinformatics Department via Amazon S3 for further processing and mapping. Normalized gene expression information in log2TPM (log 2 transcript per million reads) was generated for all mice and expression data was further analyzed to identified DEGs for: 1) BA vs. isotype control, 2) BA vs. the combination therapy, and 3) the combination therapy vs. isotype control. Genes were considered differentially expressed genes (DEGs) if they had a fold change of 1.5:1 or greater and an FDR corrected p-value of 0.05 or less (p-values were adjusted across all genes passing the TPM cutoff).

Overlapping Gene Signature and Gene Set Enrichment Analysis (GSEA)

DEGs identified between treatment groups were evaluated using the "investigate gene sets" function at the Molecular Signature Database (MSigDB v7.3; Broad Institute, MIT; UC San Diego; https://www.gsea-msigdb.org/gsea/msigdb/index.jsp) to compute overlaps between the list of genes and gene sets in MSigDB, which is a collection of curated gene signatures for the evaluation of biological functions. GSEA software (v4.1.0; Broad Institute, MIT; UCSD) was used to identify enriched signaling pathways and biological functions associated with a specific treatment.

For GSEA, which uses the list rank information without using a predefined threshold to identify enriched gene signatures, the complete set of gene expression values in log2TPM was

used as input for the comparison between BA treatment and the combination therapy of anti–PD-L1 and TGF-β trap. Expression differences of all genes between the two treatment groups were computed by the GSEA software, ranked based on their magnitude and statistically significance, followed by the identification of gene sets enriched in either of the treatment groups. The hallmark (H) and curated (C2) gene sets in MSigDB were used as the source of gene expression signatures. GSEA was run using a weighted scoring scheme, along with 1,000 gene set based permutation. Size of gene sets were set to be greater than 15 genes and smaller than 500 genes.

Single-cell RNAseq (scRNAseq) Analysis

scRNAseq was performed by MedGenome Inc using the 10X Genomics Chromium platform. In brief, the cryopreserved single cell samples were rapidly thawed, washed and loaded into the Chromium system targeting 5,000 cells. Following the manufacturer's instructions, barcoded sequencing libraries were generated using Chromium Single Cell 3′ v2 Reagent Kits and sequenced across 8 lanes on a HiSeq 4000 platform targeting 100,000 reads per cell. Sequencing data were aligned to the human reference genome (GRCh38) and processed using an internal scRNAseq pipeline. The raw gene expression matrix was obtained by running a Kallisto bus-based pipeline. The presence of low-quality cells or empty droplets, cell doublets or multiplets that may exhibit an aberrantly high gene count and the percentage of reads that map to the mitochondrial genome (low-quality/dying cells often exhibit extensive mitochondrial gene content) was checked.

Initial QC on the entire dataset was performed using the following criteria- (a) include features (or genes) detected in at least 10 cells, (b) include cells where at least 200 features or genes are detected to remove low quality or empty droplets, and (c) remove from the main analysis cells with >5000 unique molecular identifier (UMI) counts; <200 genes and >3,000 genes. From the filtered cells (with no mitochondrial gene detected), the expression matrix was then

"logNormalized" which normalizes the gene expression measurements for each cell by the total expression, multiplies this by a scale factor (10,000 by default), and log-transforms the result. Each feature (gene) in the dataset was then scaled and centered. Variable genes were selected as the top 2000 highly variable genes expressed by more than 0.1% of cells in each sample. A PCA (Principal Components Analysis) based dimensionality reduction was calculated using the most variable features obtained from the step above. A k-nearest neighbor graph was constructed and then clustered using the Louvain algorithm to find clusters of cells in the dataset. The UMAP (Uniform Manifold Approximation and Projection) dimension reduction technique was utilized for visualization using the first 50 principal components (PCs).

A visualization algorithm that produces clustering trees (clustree) was used to visualize the clusters of samples at different resolutions. This helps us in understanding how samples move as the number of clusters increases and then deciding on the optimum resolution for clustering a particular dataset. Next, annotation of cell clusters was performed by comparing the expression levels of canonical markers for different cell lineages (PanglaoDB - Single cell sequencing resource for gene expression data, https://panglaodb.se) and by comparing the DEGs computed between two groups cells using the MAST algorithm. A quantitative method was devised to assign cell types based on the average expression and percentage of expression of a group of cell type markers (genes) in any cluster. To call a cluster of a particular cell type, the average expression of the group of markers should be at least greater than 1 unit and the percentage of expression must be at least greater than 30. These criteria were used to identify cells belonging to a variety of lineages and to assess the change in cell populations with differences in treatment. To investigate the effect of each treatment type on the composition of cell population in the TME, percentages of change in cell types were calculated (count of individual cell types/ sum of the count of all cell

types per sample per treatment group). DEGs in treatment groups with respect to the isotype control group was calculated using the MAST algorithm, retaining genes detected in at least 0.1% of the cells in either group being compared.

Immunohistochemistry staining on mouse tissue samples

For sequential chromogenic double staining of CD68 and iNOS/Arg-1, MC38 tissue samples were deparaffinized, washed, and antigen retrieval was performed using a citrate-based epitope retrieval solution at pH 6 on the BOND-RX autostainer (Leica Microsystems). After blocking, slides were stained with an anti–mouse CD68 antibody (Abcam, ab125212) followed by the incubation of a HRP polymer. Subsequently, a stripping step was performed by using citrate buffer for 20min at 100°C. Tissue slides were then stained with anti–mouse Arginase-1 antibody (Cell Signaling Technology, 93668) or anti–mouse iNOS antibody (Cell Signaling Technology, 13120) followed by the incubation of an AP polymer. Double stained slides were washed, and signals were visualized using the DAB and Fast Red chromogen. Masson's Trichrome staining was performed using the Trichrome Stain Kit (Abcam, ab150686) according to the manufacturer's protocol. Dehydrated and mounted slides were scanned using a Hamamatsu NanoZoomer slide scanner. Slide images were evaluated with the software programs Aperio ImageScope (Leica Biosystems) and ImageJ (National Institutes of Health) with the immunohistochemistry toolbox plug-in.

Multiplex immunofluorescence staining

Human formalin-fixed, paraffin-embedded lung tumor tissue sections were deparaffinized, washed, and treated with citrate-based epitope retrieval solution on the Leica Bond RX autostainer (Leica Biosystems). Next, sections were incubated with Fc receptor blocker (Innovex Biosciences, NB309) and then 5% normal donkey serum at room temperature for 30 min. Sections were

incubated at 4 °C overnight with the following primary antibodies: PD-L1 (Abcam, 228462), TGF-β1 (LSBio, LS-B4772), α-smooth muscle actin (α-SMA) (Invitrogen, PA5-18292), F4/80 (Invitrogen, MA1-91124). The next day, tissue samples were washed and incubated with secondary antibodies at room temperature for 1 hour. Secondary antibodies used were: Donkey anti-Rabbit IgG Alexa Fluor Plus 488 (Invitrogen, A32790), Donkey anti-Mouse IgG Alexa Fluor 594 (Invitrogen, A21203), Donkey anti-Goat IgG Alexa Fluor 647 (Invitrogen, A21447), Donkey anti-Rat IgG Alexa Fluor Plus 647 (Invitrogen, A48272TR). Tissue slides were incubated with Hoechst 33342 (Invitrogen, H3570) as a counterstain and cover-slipped with Prolong Gold antifade reagent (Invitrogen, P36934). The images were taken using a Plan Apo 60X oil immersion objective on a Nikon C2 confocal coupled to a Nikon Eclipse Ti microscope.

Cell Binding Assay

HEK293-PD-L1 cells were pre-blocked with isotype control (60 μg/mL, 2X) with or without recombinant human TGF-β1 (260 ng/mL, 2X) for 30 minutes on ice. For TGF-β1 pre-blocking samples, biotinylated TGF-β1 (1:1 serial dilution starting from 11.76 μg/mL, 4X, with 8 concentrations) were pre-mixed with BA or TGF-β trap (36 μg/mL, 4X) for 30 minutes on ice and then the cells were incubated with the mixture of antibody/biotinylated TGF-β1 on ice for 30 minutes. For samples without TGF-β1 pre-blocking, cells were incubated with BA or inactive anti–PD-L1 (36 μg/mL, 4X) for 15 minutes on ice and then biotinylated TGF-β1 (1:1 serial dilution starting from 11.76 μg/mL, 4X, with 8 concentration) was added. The cells were further incubated on ice for 30 minutes. After washing, cells were incubated with anti–human IgG AF488 (2.5 μg/mL) and streptavidin (SA)-APC (0.67 μg/mL) for 30 minutes on ice. Cells were then washed and re-suspended in 1% BSA PBS with 7AAD and analyzed by flow cytometry (LSR II). The data were processed by Flowjo V10 and graphed by Graphpad Prism V8.

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BA-induced TGF-β Depletion Assay

HEK293 parental and HEK293-PD-L1 cells were incubated with 150 ng/mL TGF-β trap or BA and 100, 50, 25, or 12.5 ng/mL biotinylated TGF-β1 for 2 days. Biotinylated TGF-β1 in the medium was captured by plate bound streptavidin (1 μg/well of 96-well plate), saturated by adding 10 μg/mL TGF-β trap or BA, and detected by HRP conjugated anti–hIgG1 secondary antibody (80 ng/mL). To block the binding of BA to PD-L1, cells were pre-incubated with 3 μg/mL anti–PD-L1 antibody for 30 minutes. Biotinylated TGF-β1 in the culture supernatant at 48 hours was measured by a sandwich ELISA assay.

PD-L1 Dependent Cell Binding Assay

4T1-pSmad Luc cells were pre-treated with 1:2 serial diluted IFN-γ (0.005~ 10 ng/mL) for 24 hours to induce various levels of PD-L1 expression. Cells were pre-incubated with human IgG1 (inactive anti–PD-L1) (60 μg/mL, 2X) and TGF-β1 (260 ng/mL, 2X) for 30 minutes on ice. BA, anti–PD-L1, or TGF-β trap (36 μg/mL, 4X) were pre-mixed with 5.88 μg/mL (4X) biotinylated TGF-β1 and incubated for 15 minutes on ice. The antibody/biotinylated TGF-β1 mixture was added to the cells and incubated for 30 minutes on ice. After washing, anti–human IgG1 AF488 (2.5 μg/mL) and SA-APC (0.67 μg/mL) were added, and cells were incubated for 30 minutes on ice. Cells were washed and resuspended in 1% BSA PBS with 7AAD. Cells were collected by flow cytometry (LSR II), and the data were processed by Flowjo V10 and graphed by Graphpad Prism V8.

Titration of BA and biotinylated TGF-β1 in cell binding assay

HEK293-PD-L1 cells were pre-blocked with human IgG1 (inactive anti–PD-L1) (60 μg/mL, 2X) and TGF-β1 (260 ng/mL, 2X) for 30 minutes on ice. Serial dilutions of biotinylated TGF-β1 (1:1, 0.367~47 μg/mL, 4X) were pre-mixed with BA, anti–PD-L1, TGF-β trap (36 μg/mL,

4X) for 15 minutes on ice, and cells were then incubated with 1:2 serial diluted BA/TGF-β trap (0.02~54 μg/mL, 2X) or the mixture of BA/TGF-β trap with biotinylated TGF-β1 for 30 minutes on ice. Anti–human IgG1 AF488 (2.5 μg/mL) with or without SA-APC (0.67 μg/mL) was added, and cells were incubated for an additional 30 minutes on ice before being resuspended in 1% BSA PBS with 7AAD for flow cytometry analysis (LSR II).

Internalization Assay

To investigate internalization of BA, HEK293-PD-L1 cells were pre-blocked with human IgG (60 μg/mL, 2X) and TGF-β1 (260 ng/mL, 2X) for 15 minutes on ice. BA, anti–PD-L1, inactive anti–PD-L1 or TGF-β trap (36 μg/mL, 4x) were pre-mixed with biotinylated TGF-β1 (5.88 or 23.6 μg/mL, 4x) and incubated for 15 minutes on ice. The biotinylated TGF-β1/antibody mixture, AF488 conjugated BA or AF488 conjugated anti–PD-L1 antibodies (18 μg/mL, 2X) were added to the cells and incubated for 30 minutes on ice. After washing, SA-AF488 (6.67 μg/mL) in ice cold 1% BSA PBS was added, and cells were incubated for 20 minutes on ice. Cells were then incubated on ice (as control) or at 37°C (for internalization) for 4 or 20 hours. To quench cell surface binding, cells were incubated with anti–AF488 (25 μg/mL) on ice for 30 minutes. Cells were washed and resuspended in flow buffer with 7AAD and were collected by flow cytometry (LSR II). The data were processed by Flowjo V10 and graphed by Graphpad Prism V8.

Colocalization Assays

Two methods were used to investigate colocalization. Method 1: HEK293-PDL1 cells were stained with HOECHST (10 μg/mL) in 1% BSA PBS for 15 minutes at room temperature, washed, and blocked with blocking mix (30 μg/mL human IgG and 130 ng/mL TGF-β1) on ice for 15 minutes. Antibodies (10 μg/mL) and biotinylated TGF-β1 (5.88 μg/mL) were pre-mixed on ice for 15 minutes, then added to cells and incubated on ice for 30 minutes. Cells were then stained

on ice for 20 minutes with SA-AF488 (5 µg/mL) and pHrodo conjugated anti–human IgG (10 µg/mL). After washing, cell medium was replaced with warm medium (without phenol red) and internalization of antibodies was recorded by CellDiscoverer 7 (x20) every 30 min for 20 hours, in 37°C and 5% CO_2.

Method 2: HEK293-PDL1 cells were treated or not treated for 8 hours with BA alone (10 µg/mL), TGF-β1 alone (5.88 µg/mL), or BA plus TGF-β1 (10 µg/mL, 5.88 µg/mL respectively). Following treatment, cells were fixed with 4% PFA and permeabilized using Triton-X100. Cells were then stained with DAPI (1 µg/mL), anti–TGF-β1 AF488 (1.2 µg/mL), anti–LAMP2 AF594 (5 µg/mL), and anti–human LC (lambda) APC (1.66 µg/mL). Images were taken with a Leica SP5 Confocal Microscope (x63) and analyzed by FIJI software.

Decay Assay

To measure biotinylated TGF-β1 degradation, HEK293-PD-L1 cells were pre-blocked with human IgG (60 µg/mL, 2X) and TGF-β1 (260 ng/mL, 2X) for 15 minutes on ice. BA, anti–PD-L1, isotype control, or TGF-β trap (36 µg/mL, 4x) were pre-mixed with biotinylated TGF-β1 (23.6 µg/mL, 4x) (1:4 molecular ratio) for 30 minutes on ice. The biotinylated TGF-β1/antibody mixture was added, and cells were incubated for 30 minutes on ice. Following a wash, SA-AF488 (6.67 µg/mL) in 1% BSA PBS was added, and cells were incubated for 30 minutes on ice. To measure antibody degradation, HEK293-PD-L1 cells were pre-blocked with human IgG (60 µg/mL, 2X) for 15 minutes on ice. The AF488 conjugated BA, anti–PD-L1, isotype control, or TGF-β trap (18 µg/mL, 2X) was added, and cells were incubated for 30 minutes on ice. Cells were washed and resuspended in culture medium with or without 25 µM chloroquine diphosphate or 0.1% Sodium Azide. AF488 decay was measured by IncuCyte Zoom (x20) every 12 hours for 3 days (n=3).

Statistical Analysis

Binding affinities were derived from dose response curves that were generally analyzed (Nanotemper software MO Affinity Analysis) with a four-parameter fit to determine the K_D value. In cases of apparent binding affinities at or below the concentration of labelled TGF-β1, a Hill fit was used. Mass photometry data were analyzed with the Refeyn Discovery MP software. For each molecular species, the mass histogram was analyzed with a Gaussian fit, and the center mass was considered as the apparent molecular weight. The absolute mass values were obtained by referencing to a molecular weight standard (NativeMark Unstained Protein Standard-5, Life Technologies). Electron microscopy particles were manually picked for BA and BA/TGF-β1-dimer complex respectively. Picked particles were subjected to multivariate statistical analysis yielding 50 classes. The best class averages were then selected based on the quality of the signal-to-noise ratio for comparison. For IFN-γ production in the two-way MLR assay and immune profile flow cytometry analysis, p values were calculated using a one-way ANOVA with Dunnett's multiple comparisons test. A Kaplan-Meier plot was generated to show survival in MC38 tumor-bearing mice by treatment group, and significance was assessed by log-rank (Mantel-Cox) test.

For gene expression analysis, genes were considered DEGs if they had a fold change of 1.5:1 or greater and an FDR corrected p value of 0.05 or less (p values were adjusted across all genes passing the TPM cutoff). For the computation of overlaps between gene sets, p values were calculated from the hypergeometric distribution for (k-1, K, N - K, n) where k is the number of genes in the intersection of the query set with a set from MSigDB, K is the number of genes in the set from MSigDB, N is the total number of gene universe, and n is the number of genes in the query set. FDR q-values were calculated after correction for multiple hypothesis testing according to Benjamini and Hochberg.

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Detailed mathematical and statistically method employed by the GSEA was previously described[2]. In brief, p values for enrichment scores were calculated based on the Kolmogorov-Smirnov statistic, and FDR adjusted p values were calculated after correction for multiple hypothesis testing. The statistical significance of gene signature score differences between treatment groups was determined with a one-way ANOVA with Sidak's multiple comparisons test. For scRNAseq, percentages of change in cell types were calculated (count of individual cell types/ sum of the count of all cell types per sample per treatment group). DEGs in treatment arms with respect to the G1 isotype control group was calculated using the MAST algorithm, retaining genes detected in at least 0.1% of the cells in either group being compared.

SUPPLEMENTARY TABLES

Supplementary Table S1. Binding affinities of bintrafusp alfa (BA), fresolimumab, and control molecules BA one-leg, fresolimumab Fab, and fresolimumab one-arm using different methods: Surface Plasmon Resonance (SPR), Isothermal Titration Calorimetry (ITC), Micro-Scale Thermophoresis (MST).

Method	Molecule	K_D (nM)		
		TGF-β1	TGF-β2	TGF-β3
SPR [3]	Fresolimumab	1.7 ± 0.6	3.0 ± 1.2	2.0 ± 1.2
SPR	Fresolimumab	1.7	1.8	0.7
ITC	Fresolimumab	≤ 2*) x)	-	-
ITC	Fresolimumab Fab	≤ 2*) x)	-	-
MST	Fresolimumab Fab	1.6 ± 0.4+)	-	-
ITC	Fresolimumab one-arm	4.0 ± 1.0*)	-	-
SPR	BA	3.6	ND	0.4
ITC	BA	≤ 2*) x)	-	-
MST	BA	0.055 ± 0.008+)	-	-
ITC	BA one-leg	8.3 ± 3.5*)	-	-
MST	BA one-leg	4.3 ± 2.9#)	-	-

ND, not detected.
Row 1 - SPR values from Moulin et al. 2014;23(12):1698-707.
Cells with dashes (-) indicate that the experiment was not performed. +) Average of two independent measurements; #) Average of two independent measurements each at three different integration times; *) Average of three independent measurements; x) Beyond ITC differentiation limit, estimation for the upper bound for KD value.

Supplementary Table S2. Gene sets in the molecular signature database that overlap with the BA-specific 72 DEG list

Gene set name [# of genes (K)]	Description	# of genes in overlap (k)	p value	FDR q-value
Hallmark_Epithelial_mesenchymal_transition [200]	Genes defining epithelial-mesenchymal transition, as in wound healing, fibrosis and metastasis	6	$4.84E^{-7}$	$8.44E^{-5}$
NABA_Matrisome [1026]	Ensemble of genes encoding extracellular matrix and extracellular matrix-associated proteins	17	$5.82E^{-14}$	$3.25E^{-10}$
Reactome_Extracellular_matrix organization	Extracellular matrix organization	8	$1.33E^{-8}$	$6.75E^{-6}$
Reactome_Collagen_formation [90]	Collagen formation	4	$9.44E^{-6}$	$2.19E^{-3}$

FDR: False Discovery Rate, NES: Normalized Enrichment Score

Supplementary Table S3. Gene sets in the molecular signature database that overlap with the 530 BA upregulated DEG list

Gene set name [# of genes (K)]	Description	# of genes in overlap (k)	p value	FDR q value
Tarte_Plasma_cell_vs_plasmablast_DN [307]	Genes downregulated in mature plasma cells compared with plasmablastic B lymphocytes.	43	$4.52E^{-30}$	$2.52E^{-27}$
Matsuda_Natural_killer_differentiation [479]	Genes changed between developmental stages of Valpha14i NKT cells	44	$4.26E^{-23}$	$8.81E^{-21}$
Reactome_Cytokine_signaling_in_immune_system [858]	Cytokine Signaling in Immune system	53	$1.16E^{-19}$	$1.47E^{-11}$
Li_Induced_T_to_natural_killer_UP [316]	Genes up-regulated in ITNK cells (T-lymphocyte progenitors [DN3 cells] reprogrammed to NK cells by ablation of BCL11B gene), compared with the parental DN3 cells	32	$2.33E^{-18}$	$2.41E^{-16}$

FDR: False Discovery Rate, ITNK: Induced-T cell like natural killer, NK: Natural killer, NKT: Natural killer T lymphocyte

Colocalized inhibition of TGF-β and PD-L1

Supplementary Table S4. Gene sets in the molecular signature database that overlap with the 603 BA downregulated DEG list

Gene set name [# of genes (K)]	Description	# of genes in overlap (k)	p value	FDR q value
Chandran_Metastasis_UP [207]	Genes upregulated in metastatic tumors from the whole panel of patients with prostate cancer	21	$1.81E^{-11}$	$6.32E^{-9}$
Boquest_Stem_cell_UP [262]	Genes up-regulated in freshly isolated CD31- [GeneID=5175] (stromal stem cells from adipose tissue) versus the CD31+ (non-stem) counterparts	23	$3.87E^{-11}$	$1.27E^{-8}$
NABA_Matrisome [1026]	Ensemble of genes encoding extracellular matrix and extracellular matrix-associated proteins	43	$7.89E^{-9}$	$1.43E^{-6}$
Koinuma_Targets_of_SMAD2_or_SMAD3 [843]	Genes with promoters occupied by SMAD2 or SMAD3 [GeneID=4087, 4088] in HaCaT cells (keratinocyte) according to a ChIP-chip analysis	34	$7.13E^{-7}$	$4.91E^{-5}$

FDR: False Discovery Rate

Supplementary Table S5. Key enriched gene sets identified by GSEA between BA and the combination treatment group

Treatment comparison	Gene set	Set size	NES	p value	FDR q value
BA vs. combination therapy (anti–PD-L1 and TGF-β trap)	Biocarta_IL12 pathway	16	2.11	<0.001	0.013
	PID_CD8_TCR_downstream_pathway	46	2.06	<0.001	0.015
	Mori_Immature_B_lymphocyte_DN	86	1.90	<0.001	0.047
	Li_induced_T_to_natural_killer_up	257	1.86	<0.001	0.059
	Reactome_Downstream_TCR_signaling	77	1.85	<0.001	0.060
	Goldrath_Antigen_response	325	1.83	<0.001	0.062
	Hallmark_Interferon_gamma_response	179	1.69	<0.001	0.011
	NABA_Core_matrisome	114	-2.09	<0.001	0.006
	Anastassiou_Multicancer_invasiveness_signature	52	-2.00	<0.001	0.008
	NABA_ECM_glycoproteins	82	-1.88	0.001	0.052
	Ivanova_Hematopoiesis_stem_cell	128	-1.73	<0.001	0.124
	NABA_Collagens	22	-1.66	0.017	0.168
	Hallmark_TGF_β_signaling	47	-1.59	0.016	0.216

FDR: False Discovery Rate, NES: Normalized Enrichment Score, TCR: T cell receptor, TGF-β: Transforming growth factor beta

Colocalized inhibition of TGF-β and PD-L1

Supplementary Table S6. Key lineage markers used for annotation of cell clusters identified in single-cell transcriptome

Cell type	Marker gene
T cells	Ccr7, Cd2, Cd3d, Cd3e, Cd3g, Cd4, Cd7, Cd8a, Eomes, Foxp3, Pdcd1, Sell, Tbx21, Trdc
B cells	Cd19, Cd20, Cd22, Cd27, Cd38, Cd40, Cd79a, Cd79b, Cd80, Ighd, Ighg1, Ighgm, Il6, Itgax, Pdcd1, Ptprc, Sdc1, Tnfrsf17
Tregs	Cd4, Ctla4, Eomes, Foxp3, Ikzf2, Il10, Il2, Il2ra, Lag3, Tbx21, Tngrsf4
Macrophages	Adgre1, Arg1, Cd11c, Cd163, Cd200r1, Cd68, Cd86, Csf1r, Fizz1, Itga2, Itgal, Itgam, Mgl2, Mrc1, Nrp2, Pdcd2, Spp1, Tlr2, Tlr4
NK cells	Cd127, Cd16, Cd3d, Cd56, Cd69, Eomes, Itga2, Itgam, Klrb1b, Klrd1, Klrk1, Ncr1, Nkg7, Tbx21
Fibroblasts	Acta2, Bgn, Bmp2, Bmp5, Cav1, Cd70, Cd74, Chrne, Col1a1, Col3a1, Col5a1, Fap, Fstl1, Pdgfra, Postn, S100a4, S100b, Sox10, Tagln, Wnt5a
Myeloid cells	Adgre1, Arg1, Cd14, Cd163, Cd164, Cd33, Cd36, Cd38, Cd68, Cd80, Fcgr3, Fut4, Itgam, Krt19, Krt20, Ly6g, Mafb, Mertk, Mki67, Mrc1, Olr1, S100a4, S100a8
Mast cells	Cd13, Cd200r3, Cd63, Cma1, Fcer1g, Hrh1, Hrh2, Kit, Kitl, Ptgds
Dendritic cells	Cd11c, Cd14, Cd34, Cd83, Clec9a, Csf2, Flt3, Il12, Klk1, Siglech, Thbd, Tlr4, Tnfa, Vegf
Stromal cells	Acta2, Bst1, Cd40, Cd80, Col3a1, Dcn, Eng, Epsti1, Mmp9, Myh11, Postn, Ptprc, Stag3, Synpo2, Tagln, Thy1, Wnt5a
Epithelial / endothelial cells	Anks3, Bcl11b, Cd2ap, Cd34, Cdh1, Ceacam1, Cldn1, Col1a1, Cxcl14, Egr1, Epcam, Fat1, Fgfr3, Icam1, Itga5, Itgal, Klf9, Krt1, Krt10, Krt19, Krt5, Lyve1, Muc1, Neph1, Neph2, Ntrk2, Pecam1, Slc12a1, St6gal1, Vwf

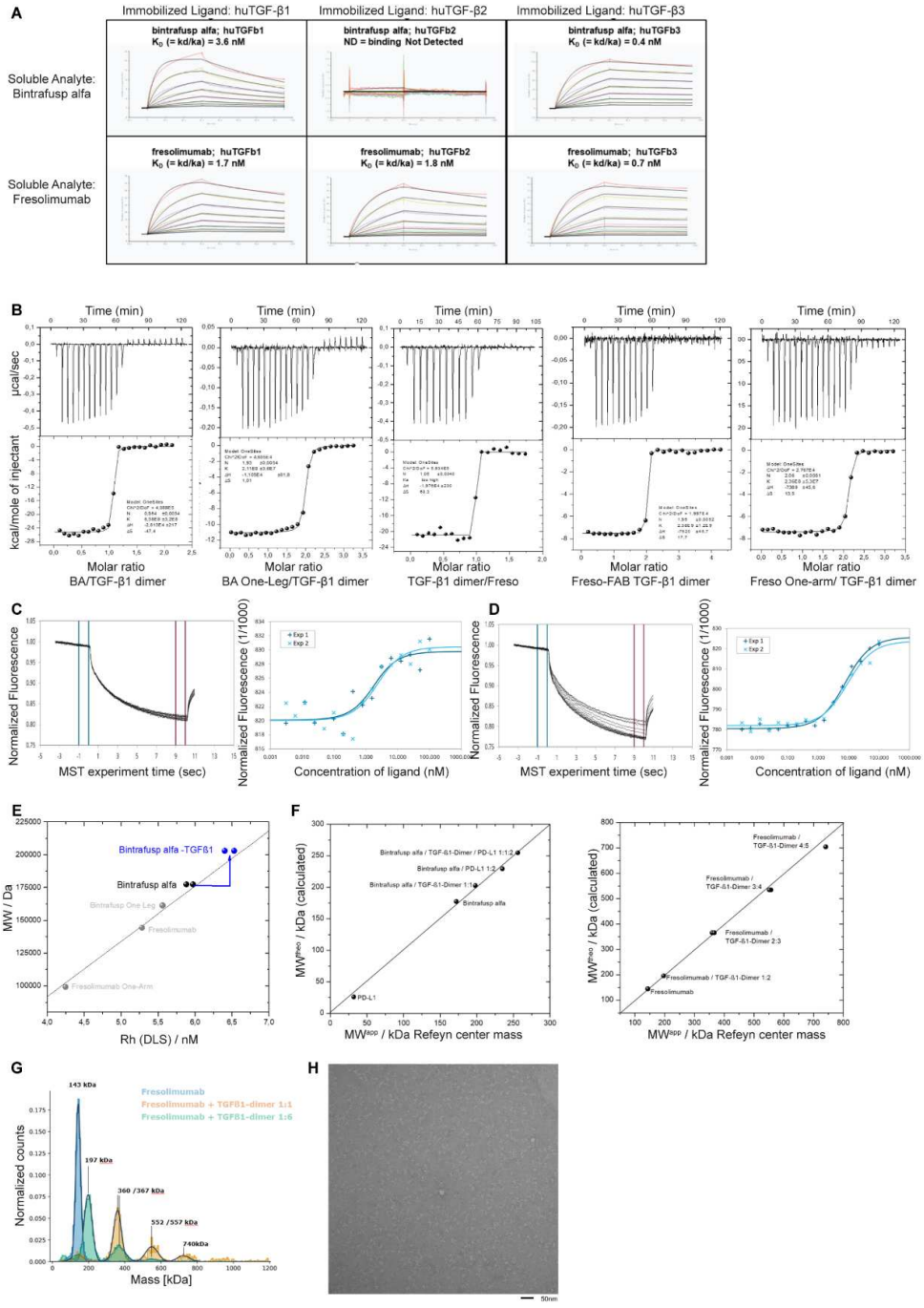
Supplementary Table S7. Key enriched gene sets identified in single-cell transcriptome by GSEA between BA and the anti–PD-L1 and TGF-β trap combination therapy

Cell type	Gene set	Set size	NES	p value	FDR q value
Tregs	Hallmark_TNFa_signaling_via_NFkB	75	-1.64	0.001	0.009
	Hallmark_Hypoxia	61	-1.65	0.001	0.009
	Hallmark_Allograft_rejection	67	-1.68	0.001	0.009
	Hallmark_Interferon_gamma_response	77	-1.92	0.001	0.009
	Hallmark_Interferon_alpha_response	42	-2.07	0.001	0.009
	Hallmark_Inflammatory_response	36	-1.63	0.003	0.022
	Hallmark_Complement	48	-1.56	0.005	0.030
	Hallmark_Apoptosis	46	-1.52	0.009	0.038
	Hallmark_Kras_signaling_up	29	-1.57	0.009	0.038
	Hallmark_IL6_JAK_Stat3_signaling	23	-1.59	0.009	0.038
	Hallmark_Glycolysis	40	-1.52	0.012	0.043
	Hallmark_IL2_Stat5_signaling	37	-1.52	0.014	0.047
Fibro-blasts	Hallmark_Interferon_alpha_response	21	-2.24	0.001	0.011
	Hallmark_Interferon_gamma_response	41	-2.16	0.001	0.011
	Hallmark_TNFa_signaling_via_nfkb	30	-2.13	0.001	0.011
	Hallmark_Complement	29	-2.13	0.001	0.011
	Hallmark_Allograft_rejection	37	-2.04	0.001	0.011
	Hallmark_Estrogen_response_early	12	-1.86	0.003	0.020
	Hallmark_Inflammatory_response	20	-1.86	0.005	0.024
	Hallmark_Apoptosis	24	-1.78	0.006	0.024
	Hallmark_PI3K_AKT_MTOR_signaling	16	-1.70	0.014	0.047
	Hallmark_Oxidative_phosphorylation	65	1.61	0.009	0.03
	Hallmark_Myc_targets_v1	92	1.75	0.005	0.024

FDR: False Discovery Rate, NES: Normalized Enrichment Score

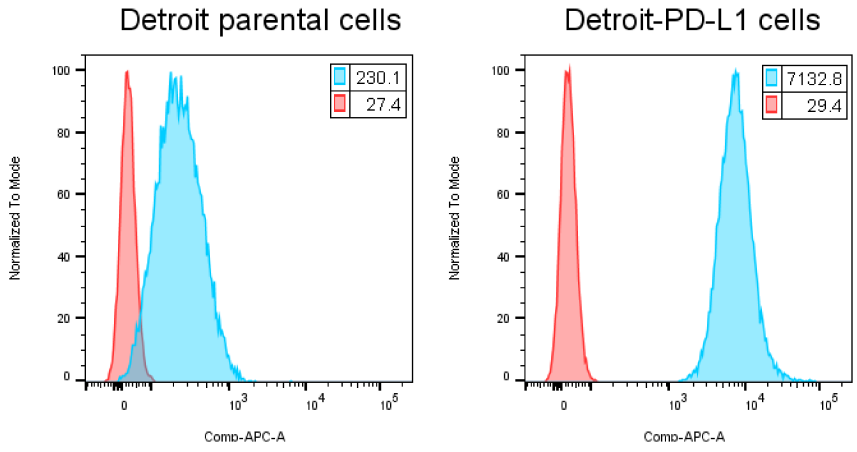
Colocalized inhibition of TGF-β and PD-L1

SUPPLEMENTARY FIGURES



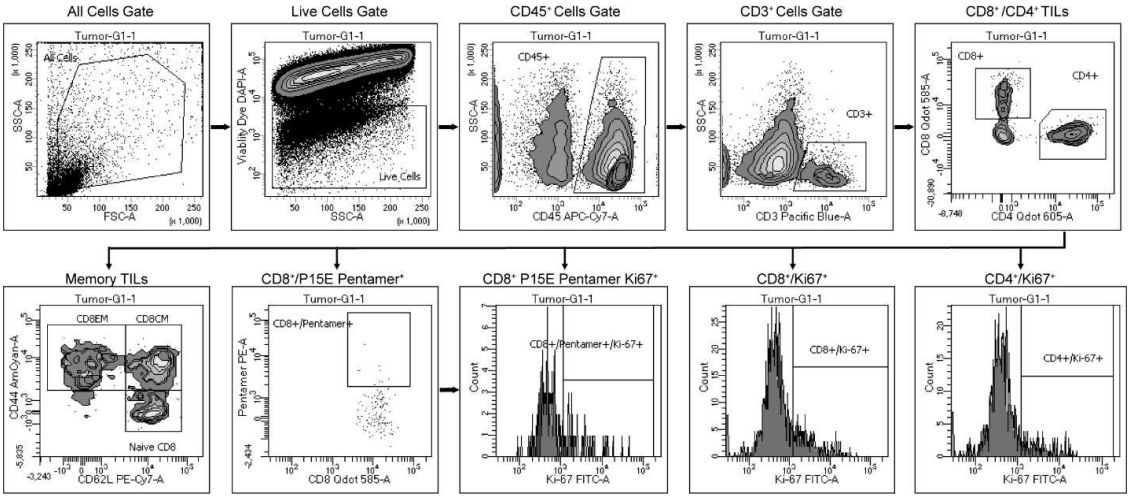
Supplementary Figure S1. (**A**) SPR sensorgram plots of soluble BA and soluble fresolimumab binding to immobilized TGF- β1, β2 and β3. Top three panels show BA binding and bottom three

panels show fresolimumab binding. First, second, and third columns show binding to immobilized TGF-β1, TGF-β2 and TGF-β3, respectively. (**B**) Exemplary ITC titrations of TGF-β1 with BA, BA one-leg, fresolimumab, fresolimumab Fab, and fresolimumab one-arm, respectively. ITC titration graphs with time-resolved heat release pulses for each injection (top panel) and binding isotherms obtained from integrated heats plotted against molar ratio of titrant to TGF-β1 dimer (bottom panel). The consistent values for stoichiometries indicate a quantitative binding functionality of the TGF-β1 preparation. (**C**) MST dose response TGF-β1 dimer (1 nM labeled) and fresolimumab Fab. Left graph: Traces exemplarily from one experiment. Blue lines indicate the area for averaging the baseline fluorescence and red lines indicate the area for averaging the fluorescence after thermal induction. Right graph: Combined dose response curve for fluorescence change from two experiments. (**D**) MST dose response for TGF-β1 (1 nM labeled) and BA one-leg. Left graph: Traces exemplarily from one experiment. Blue lines indicate the area for averaging the baseline fluorescence and red lines indicate the area for averaging the fluorescence after thermal induction. Right graph: Combined dose response curve for fluorescence change from two experiments. (**E**) Plot of measured hydrodynamic radii from DLS vs. calculated molecular weight. Black: Rh values for BA, Blue: Rh values for 1:1 a mixture of BA with TGF-β1. (**F**) Plots for the correlation of the calculated molecular weight of proteins and expected complexes thereof (see label at data point) with the corresponding experimental masses determined by mass photometry. Left panel: Mass correlation for samples with BA and complexes with PD-L1 and TGF-β1 dimer. Right panel: Mass correlation for samples with fresolimumab and complexes with TGF-β1 dimer. The diagonal line represents a 1 to 1 correlation. Note the slight deviation for higher order complexes with masses beyond 500 Da. This is most likely due to the lack of size coverage by the molecular weight standard in this range. (**G**) Overlay of mass photometry histogram plots for 10 nM fresolimumab (blue), a mixture of 10 nM fresolimumab with 10 nM TGF-β1 dimer (yellow) and a mixture of 10 nM fresolimumab with 60 nM TGF-β1 dimer (green). Each histogram species is fitted with a Gaussian function (black line) and the center masses is displayed as peak label. Note that for mixtures of fresolimumab and TGF-β1 a mixture of species is observed, and that the appearance of species is modulated by the mixing ratio. (**H**) Representative micrograph collected on negatively stained BA.

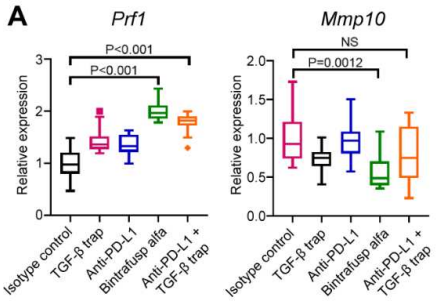


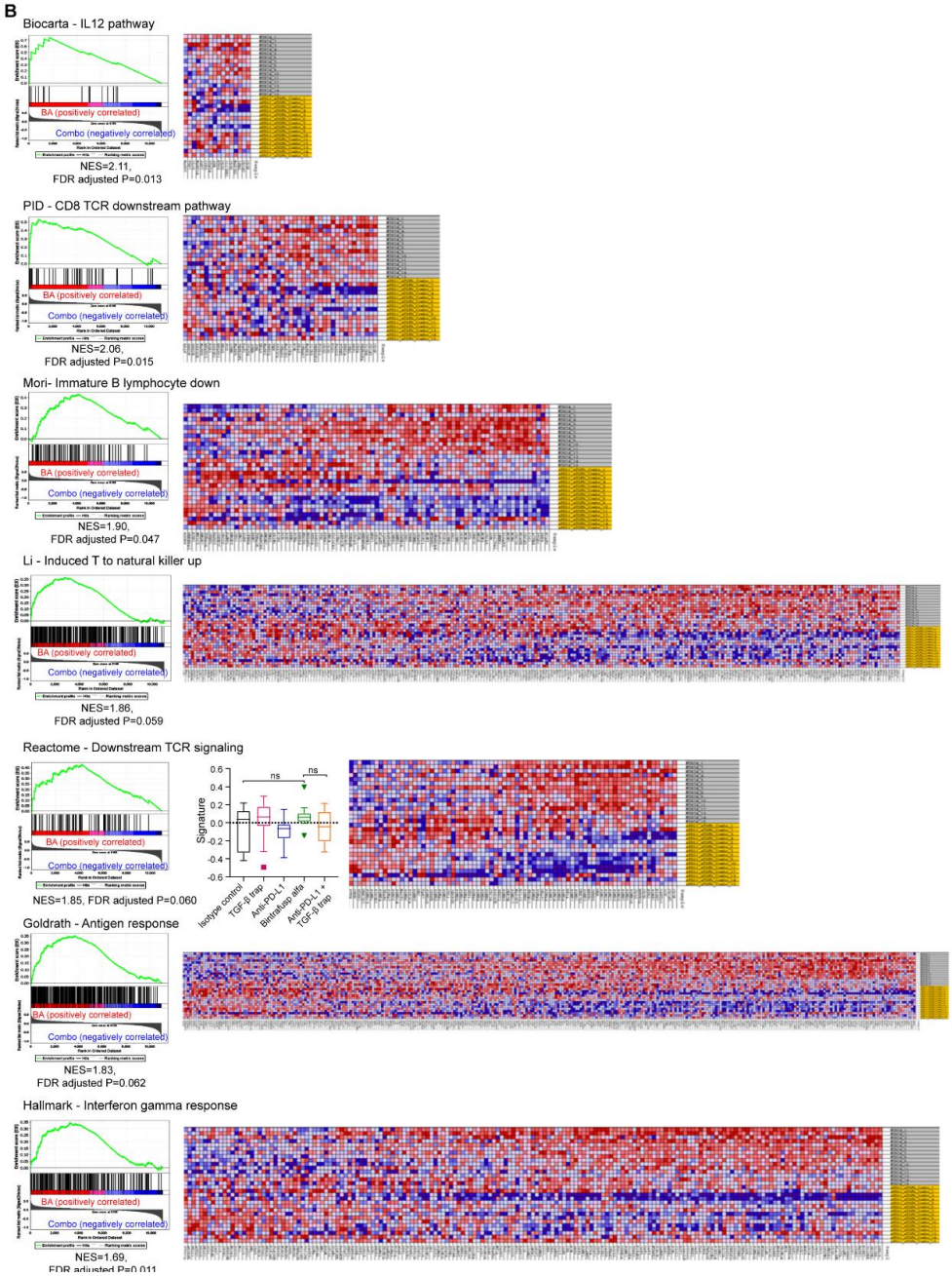
Supplementary Figure S2. Flow cytometry of PD-L1 expression in parental and PD-L1-overexpressing Detroit 562 cells.

Colocalized inhibition of TGF-β and PD-L1

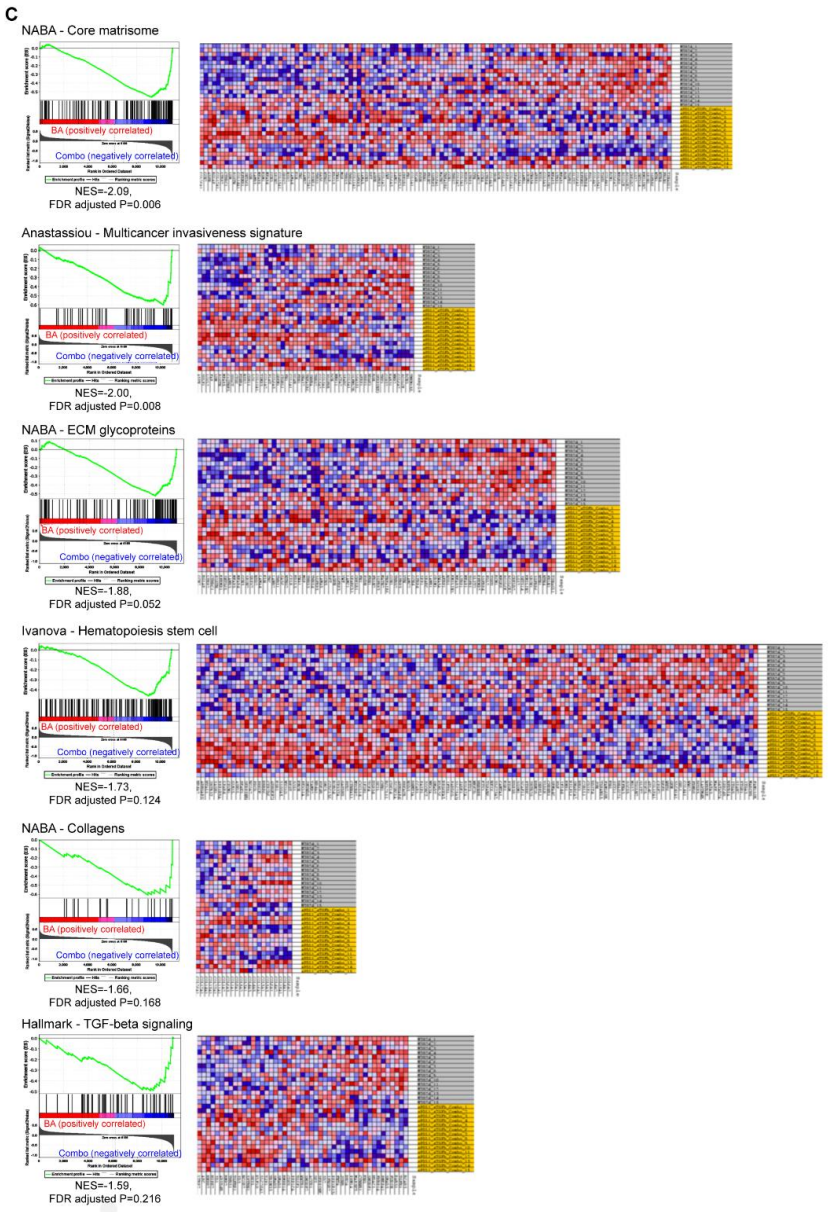


Supplementary Figure S3. Gating strategy of flow cytometry analysis of MC38 tumors. C57BL/6 mice (n = 8 per group) bearing MC38 tumors were treated iv on Day 0, 1, 2 with isotype control (400 µg), TGF-β trap (492 µg), anti–PD-L1 (400 µg), BA (492 µg), fresolimumab (200 µg), or a combination of anti–PD-L1 with TGF-β trap or fresolimumab. Tumors were harvested on Day 6 and dissociated. Cells were gated for flow cytometry analysis using the outlined strategy.




B
Biocarta - IL12 pathway
BA (positively correlated)
Combo (negatively correlated)
NES=2.11,
FDR adjusted P=0.013
PID - CD8 TCR downstream pathway
BA (positively correlated)
Combo (negatively correlated)
NES=2.06,
FDR adjusted P=0.015
Mori- Immature B lymphocyte down
BA (positively correlated)
Combo (negatively correlated)
NES=1.90,
FDR adjusted P=0.047
Li - Induced T to natural killer up
BA (positively correlated)
Combo (negatively correlated)
NES=1.86,
FDR adjusted P=0.059
Reactome - Downstream TCR signaling
BA (positively correlated)
Combo (negatively correlated)
NES=1.85, FDR adjusted P=0.060
ns
ns
Signature
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
Isotype control
TGF-β trap
Anti-PD-L1
Bintrafusp alfa
Anti-PD-L1 + TGF-β trap
Goldrath - Antigen response
BA (positively correlated)
Combo (negatively correlated)
NES=1.83,
FDR adjusted P=0.062
Hallmark - Interferon gamma response
BA (positively correlated)
Combo (negatively correlated)
NES=1.69,
FDR adjusted P=0.011

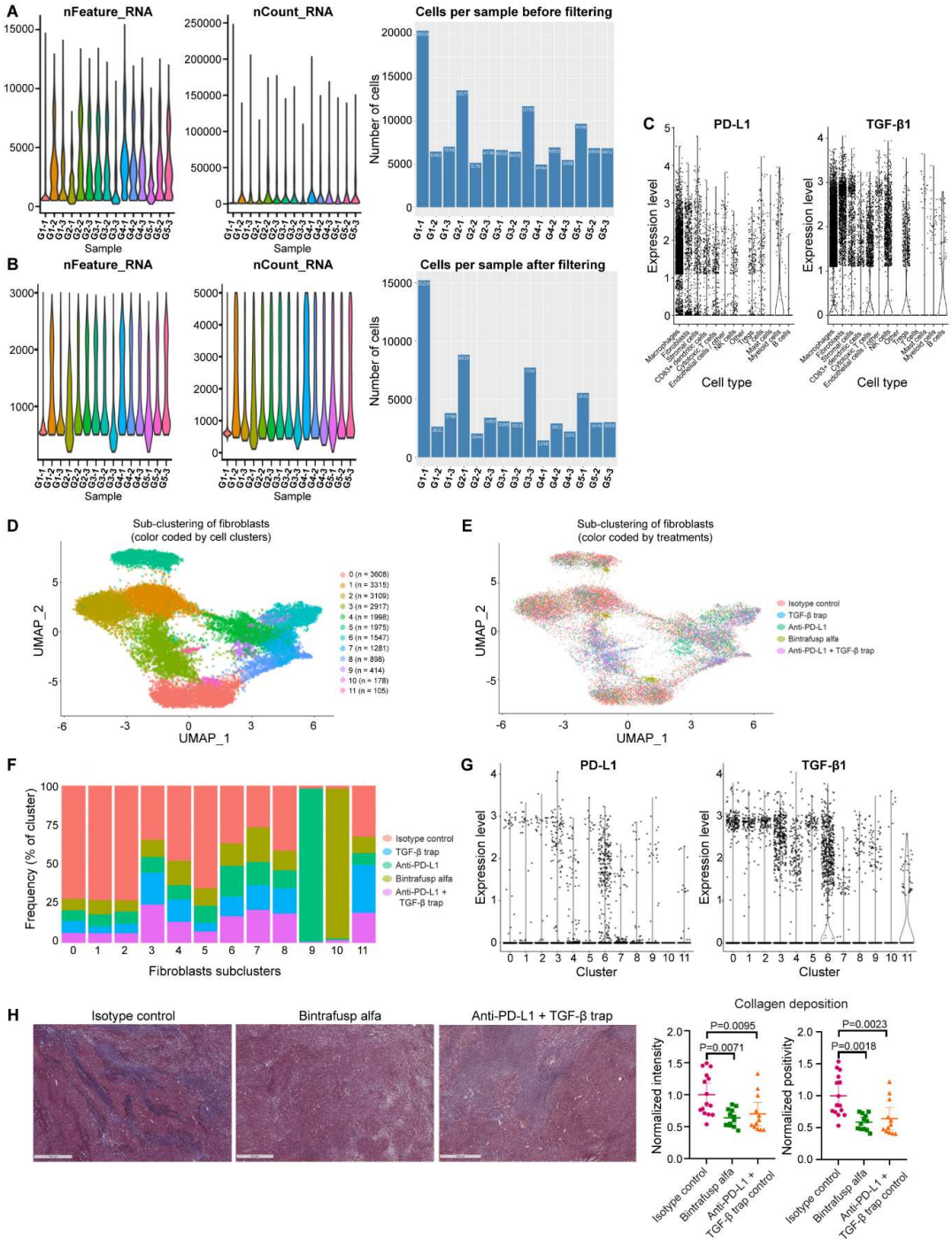
Colocalized inhibition of TGF-β and PD-L1



Supplementary Figure S4. GSEA indicates a significant increase in immune responses and a decrease in gene signatures specific to metastasis, ECM organization, collagen deposition, and TGF-β signaling activity following treatment with BA vs. anti–PD-L1 and TGF-β trap combination therapy. Mice (n=15 per group) bearing MC38 tumors were treated iv with inactive anti–PD-L1 (400 µg), anti–PD-L1 (400 µg), TGF-β trap (492 µg), BA (492 µg), or the combination therapy on Days 0, 1, and 2. Mice were sacrificed on Day 6 and RNAseq analysis was performed on tumor samples. (**A**) Gene expression of *Prf1* and *Mmp10*. Relative expression is presented as boxplots. P-values were generated with one-way ANOVA with Dunnet's multiple comparison. (**B**) Enrichment plots of gene sets enriched in BA-treated MC38 tumor-bearing mice

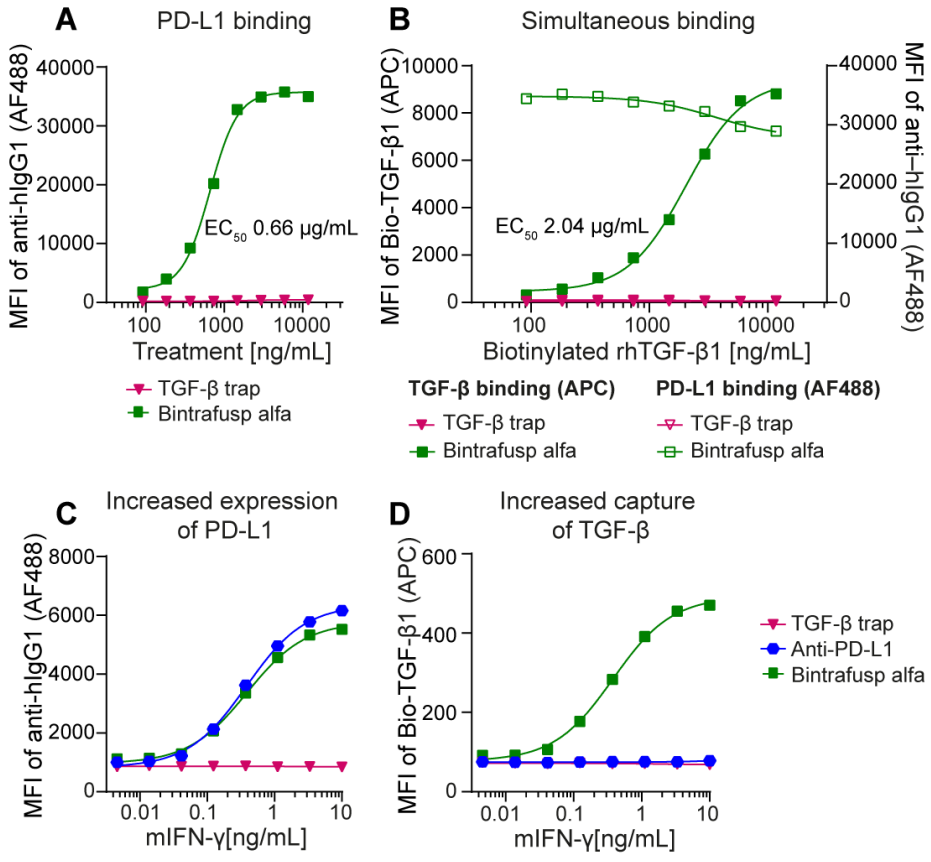
vs. the combination therapy-treated mice are presented. For the Reactome downstream TCR signature, a signature plot is also presented. (**C**) Enrichment plots of gene sets enriched in the combination therapy-treated tumor-bearing mice when compared with BA-treated mice are presented.

Colocalized inhibition of TGF-β and PD-L1



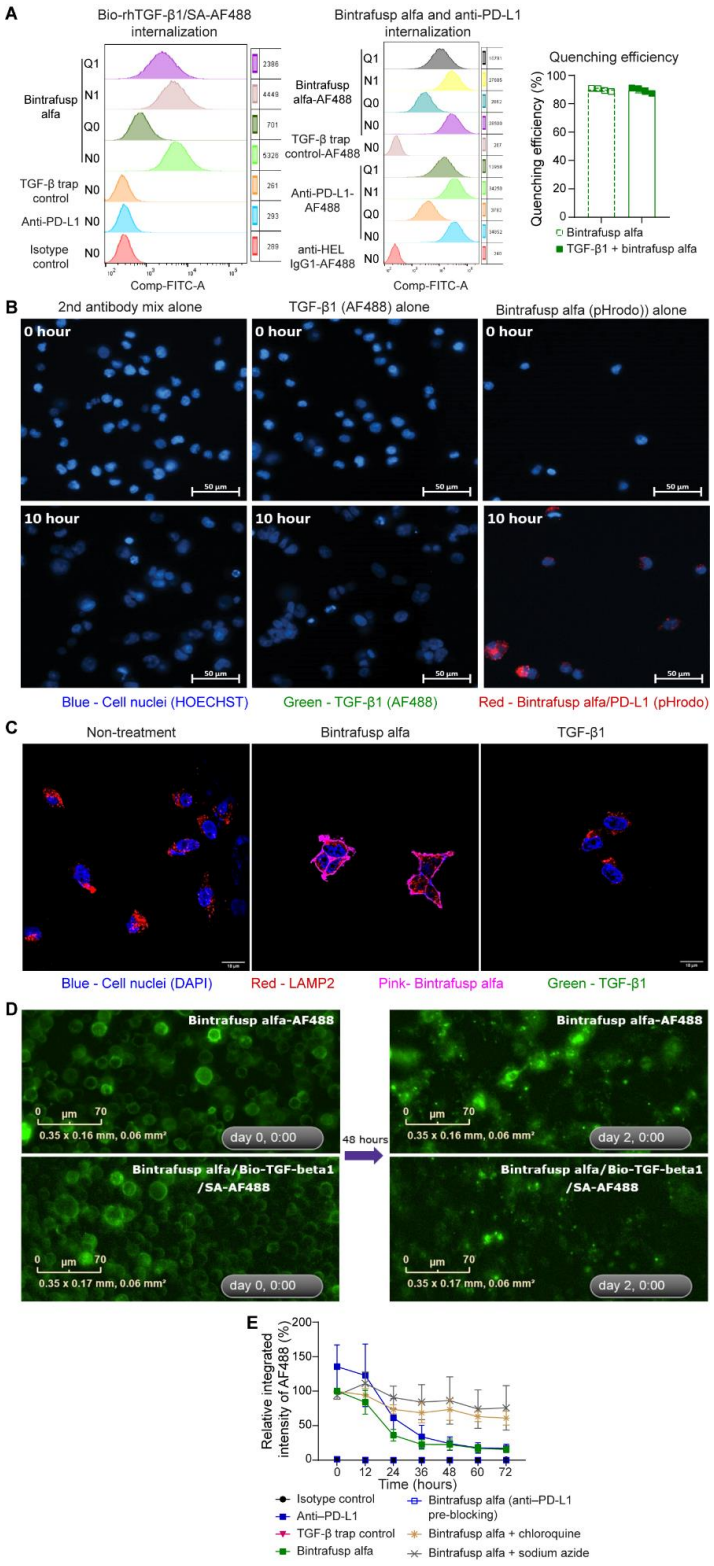
Supplementary Figure S5. Quality control of scRNAseq data and investigation of TME reprogramming by BA. Mice (n=3 per group) bearing MC38 tumors were treated as described in Figure S4 legend. Mice were sacrificed on Day 6 and tumor samples were dissociated into for

scRNAseq and histological evaluation. **(A)** The initial quality of gene expression data of all cells was evaluated. **(B)** After filtering, 125,277 cells were retrieved. The quality check retained only cells where at least 200 features were detected and at the same time included features detected in at least 10 cells, allowing bulk removal and excluding low-quality cells, empty droplets, and multiplets. 67,843 single cells with 34,319 unique genes were retained for further analysis. **(C)** Single cell expression levels of TGF-β and PD-L1 for different annotated clusters. **(D, E)** Subclustering of fibroblasts revealed 12 distinct fibroblast subclusters with most of them shared by all treatment arms. **(F)** Treatment-specific fibroblast populations were identified. Fibroblast subcluster 9 is anti–PD-L1-specific while subcluster 10 is BA-specific. **(G)** TGF-β and PD-L1 expression levels in different fibroblasts subclusters. While expression of TGF-β or PD-L1 can be detected in most fibroblast subpopulations, BA-specific subcluster 10 expresses low level of both genes. (**H**) Representative micrographs of Masson's trichrome staining and collagen deposition quantitation of MC38 tumors. Overall collagen staining intensity (blue) of the tissue section and the positivity of staining, which is the ratio between positively and negative stained area, were assessed. P-values were generated with one-way ANOVA with Dunnett's multiple comparison. Scale bar = 500 µm.



Colocalized inhibition of TGF-β and PD-L1

Supplementary Figure S6. TGF-β1 captured by BA on the cell surface of cancer cells is dependent on PD-L1 expression. **(A)** BA binding to PD-L1 on the cell surface of HEK293 PD-L1 cells was detected by AF488 conjugated anti–human IgG1 antibody by flow cytometry and the median fluorescence intensity (MFI) is shown. (**B**) The ability of BA (9 μg/mL) to capture biotinylated TGF-β1/SA-APC on the cell surface of HEK293 PD-L1 cells was measured by flow cytometry. **(C)** PD-L1 expression on the cell surface of 4T1 cells induced by IFN-γ was detected by BA or anti–PD-L1 using a secondary anti–human IgG1 antibody conjugated with AF488 and measured by flow cytometry. (**D**) Biotinylated TGF-β1 captured by BA on the surface of 4T1 cells after IFN-γ treatment was detected by SA-APC using a flow cytometry.


A
Bio-rhTGF-β1/SA-AF488 internalization
Bintrafusp alfa and anti-PD-L1 internalization
Bintrafusp alfa
TGF-β trap control
Anti-PD-L1
Isotype control
Bintrafusp alfa-AF488
TGF-β trap control-AF488
Anti-PD-L1-AF488
anti-HEL IgG1-AF488
Comp-FITC-A
Quenching efficiency
Quenching efficiency (%)
Bintrafusp alfa
TGF-β1 + bintrafusp alfa
B
2nd antibody mix alone
TGF-β1 (AF488) alone
Bintrafusp alfa (pHrodo)) alone
0 hour
10 hour
Blue - Cell nuclei (HOECHST)
Green - TGF-β1 (AF488)
Red - Bintrafusp alfa/PD-L1 (pHrodo)
C
Non-treatment
Bintrafusp alfa
TGF-β1
Blue - Cell nuclei (DAPI)
Red - LAMP2
Pink- Bintrafusp alfa
Green - TGF-β1
D
Bintrafusp alfa-AF488
Bintrafusp alfa/Bio-TGF-beta1 /SA-AF488
day 0, 0:00
day 2, 0:00
48 hours
E
Relative integrated intensity of AF488 (%)
Time (hours)
Isotype control
Anti–PD-L1
TGF-β trap control
Bintrafusp alfa
Bintrafusp alfa (anti–PD-L1 pre-blocking)
Bintrafusp alfa + chloroquine
Bintrafusp alfa + sodium azide

Colocalized inhibition of TGF-β and PD-L1

Supplementary Figure S7. TGF-β1 is internalized by BA. **(A)** One representative experiment of internalization of biotinylated TGF-β1/SA-AF488 by BA measured by flow cytometry. Left, TGF-β1 only internalized by BA but not by anti–PD-L1, inactive anti–PD-L1 or TGF-β trap; Middle, internalization of AF488 conjugated BA and AF488 conjugated anti–PD-L1; Right, quenching efficiency for membrane bound BA-AF488 or biotinylated TGF-β1/SA-AF488/BA complex. The internalization and quenching efficiency are calculated based on the MFI using the following formula. %Internalization = (1- ((N1-Q1)/(N1-N1Q0/N0))) x 100%; %Quenching efficiency (T0) = (1-(Q0-CQ0)/(N0-CN0)) x 100%. N0: no quenching at Time 0; N1: no quenching at 4 hours; Q0: quenching at Time 0; Q1: quenching at 4 hours. **(B)** Live-cell imaging of secondary antibody mix, biotinylated TGF-β1/SA-AF488, and pHrodo labeled BA at Time 0 and 10 hours (x20, control groups of Figure 7C). **(C)** Control groups of internalization/colocalization of TGF-β1 in lysosomes (Figure 7D). Time 0 is shown. AF488 conjugated anti–TGF-β1 antibody, AF594 conjugated anti–LAMP2 antibody or anti–human IgG1 APC were used to recognize TGF-β1, LAMP2 or BA, respectively. **(D)** Live-cell imaging of BA-AF488 and Bio-TGF-β1/SA-AF488 in the presence of BA was measured. Time 0 and 48 hours are shown for BA (upper panel) or TGF-β1 (lower panel). **(E)** MFI from BA-AF488 with or without chloroquine (a lysosomal inhibitor) and sodium azide (an inhibitor for receptor internalization) measured every 12 hours for 3 days. Data represent of 3 independent assays (n=3).

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