Supplementary Material

Methods

Cell lines

4T1 breast cancer, 3LL lung carcinoma, and RAW 264.7 macrophage cell lines were purchased from the ATCC. EO771 breast cancer cell line was purchased from CH3 BioSystems. MC38, MC38 OVA, and B16 OVA cell lines (under 400μg/mL G418 selection) were kindly provided by Dr. Drew Pardoll (Johns Hopkins University, Baltimore). To track tumor cells in vivo, green fluorescent protein (GFP) expressing 4T1 tumor cells were generated by transducing cells with lentiviral vector carrying the GFP gene (as a gift from Dr. Jeffrey Chao, Friedrich Miescher Institute for Biomedical Research, Basel, phage ubc nls ha pcp gfp plasmid, Addgene Plasmid #64539).

To generate CSF3 OE 4T1 cell lines, CSF3 fragments were cloned from 4T1 tumor cDNA. The following primers were used to yield CSF3 fragments: CSF3F: GGC CTT TCG ACC TGT TAA CCG CCG CCA TGG ATG GCT CAA CTT TCT GCC CAG, CSF3R: GGC GGA ATT GAT CCC GCT CGA CTA GGC CAA GTG GTG CAG AG. The CSF3 fragments and MCSV retroviral vectors with PGK promoter (MSCV-PGK-IRES-GFP, modified from MCSV-IRES-GFP, a gift from Dr. Tannishtha Reya, University of California, San Diego, Addgene, Plasmid #20672) were digested with XhoI (New England Biolabs) and assembled through Gibson assembly following manufacturer's protocol (New England Biolabs). To generate LAP OE 4T1 cell lines, the C/EBPβ (LAP) plasmid (a gift from Dr. Christopher Vakoc, Cold Spring Harbor Laboratory, Cold Spring Harbor, pcDNA3 Flag C/EBPβ LAP, Addgene, Plasmid #66979) was digested with BamHI and XhoI (New England Biolabs), gel-purification, and the fragments were ligated via Quick Ligase (following manufacturer's protocol, New England Biolabs) into retroviral expression plasmid that was predigested with BamHI and XhoI enzymes (MSCV-PGK-Myctag-IRES-GFP, modified from MCSV-IRES-GFP).

To produce retroviral supernatant, all plasmids and their corresponding empty vectors were transfected individually into a Platinum-E Retroviral Packaging Cell Line (Cell Biolabs, Inc.) by using lipofectamine

3000 (Thermo Fisher). The viral supernatants were harvested and combined at 48 and 72 hours post transfection, filtered through a SFCA 0.45μM syringe filter (Corning), and then transduced into 4T1 cells through spinofection at 2500rpm at 32℃ for 90 min.

Chemical compound

6-diazo-5-oxo-l-norleucine (DON) was purchased from Sigma-Aldrich. JHU083 (Ethyl 2-(2-Amino-4-methylpentanamido)-DON) was synthesized in house using our previously described method (25, 72).

Tumor model

4T1 cells and EO771 cells were cultured in RPMI supplemented with 10% FBS, 1% penicillin/streptomycin, and 10 mM HEPES and 3LL, RAW 264.7 and MC38 OVA cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 1% penicillin/streptomycin, and 10 mM HEPES. All cell lines were regularly tested to confirm mycoplasma free using a MycoAlert mycoplasma detection kit (Lonza). Cells were kept in culture no longer than 3 weeks. 4T1 cells (1×10^5 cells in 200 μl per mouse) were subcutaneously inoculated into the mammary fat pad of BALB/cJ mice. EO771 cells (2×10^5 cells in 200 μl per mouse) were subcutaneously inoculated into the mammary fat pad of C57BL/6 mice. MC38 (5×10^5 cells in 200 μl per mouse) or 3LL (5×10^5 cells in 200 μl per mouse) cells were subcutaneously inoculated into right flank of C57BL/6J mice. Tumor-bearing mice were treated with vehicle (PBS, referred as a NT group) or glutamine antagonist prodrug, JHU083 (1mg/kg), daily starting at day 7 or day 9 after tumor inoculation. After 7 days of 1mg/kg treatment, a lower dose (0.3 mg/kg) of JHU083 was used. On day 7, 10, 13, 17, and 24, 4T1 tumor-bearing mice were injected intraperitoneally with 250 μg anti-PD1 (InVivoPlus RMP1-14, #BP0146, BioXCell) and/or 100μg anti-CTLA4 antibodies (InVivoPlus 9H10, #BP0131, BioXCell). For the NK cell depletion study, mice were implanted with 4T1 tumor on day 0, and for NK depletion, mice were injected intraperitoneally with 100 μl of Ultra-LEAF™ Purified anti-Asialo-GM1 Antibody (Poly21460, #146002, Biolegend) on day -1 and twice a week after tumor inoculation. For TNF blocking study, 4T1 tumor-bearing mice were injected intraperitoneally with anti-TNFα antibody

(InVivoPlus, XT3.11, # BP0058, BioXCell) or rat IgG1 isotype control (InVivoPlus, HRPN, #BP0088, twice a week from day 7. Tumor burdens were monitored every 2-4 days by measuring length and width of tumor. Tumor volume was calculated using the formula for caliper measurements: tumor volume = ($L \times W^2$)/2, where L is tumor length and is the longer of the 2 measurements and W is tumor width, tumor area= $L \times W$. Mice were euthanized when tumor size exceeded 2 cm in any dimension or when the mice displayed hunched posture, ruffled coat, neurological symptoms, severe weight loss, labored breathing, weakness or pain.

To establish a pulmonary metastasis model, 4T1 cells (1×10^5 cells in 200 µl per mouse) were injected into BALB/cJ via tail vein. Tumor-bearing mice were treated with vehicle or JHU083 (1mg/kg) starting at day 2 after tumor inoculation. After 7 days of 1mg/kg treatment, a lower dose (0.3 mg/kg) of JHU083 was used. To assess MDSCs conversion into TAMs, MDSCs from the blood were isolated from CD45.1 4T1 tumor -bearing mice (21 days after 4T1 tumor inoculation). Isolated MDSCs were adoptively transferred into CD45.2 4T1 tumor-bearing mice (7 days after 4T1 tumor inoculation). Then, recipient CD45.2 4T1 tumor -bearing mice were treated with JHU083 (1mg/kg) for 7 days, then tumors were harvested.

Primary tumor and spontaneous pulmonary metastasis digestion and sorting

Tumors or lungs from tumor-bearing mice were minced in RPMI with 2% FBS, 2 mg/mL collagenase IV (GIBCO) and 20µg/mL DNase (Roche), and incubated in 37ºC for digestion. After 30 minutes, 0.02% EDTA was added to inhibit further digestion, and cells were filtered through a 70 µm cell strainer. For flow cytometry analysis, single-cell suspensions were washed with PBS, and incubated in ammonium-chloride-potassium lysis buffer to lyse contaminated erythrocytes (Quality Biological). For sorting of TAMs, the cell pellet was suspended in 30% Percoll and then carefully layered onto 70% Percoll. Samples were centrifuged at 2000 rpm at room temperature without brake for 30 minutes. After washing, tumor-infiltrating cells were isolated using a CD45 isolation kit (Miltenyl). After staining, TAMs ($CD45^+$ $7AAD^-$ $CD8^-$ $Ly6C^-$ $Ly6G^-$ $CD11b^+$ $F4/80^+$) were sorted using a BD FACSAria™ Fusion.

Flow cytometry

Single cell-suspensions were stained with antibodies after Fc blocking (anti-mouse CD16/CD32, 2.4G2, # BE0307, BioXCell). The following antibodies and staining reagents were purchased from Biolegend: anti-CD45 (30-F11, #103137), anti-F4/80 (BM8, #123141), anti-CD11b (M1/70, #101222), anti-Ly6C (HK1.4, # 128012), anti-Ly6G (RB6-8C5, #108434, #108442), anti-MHC Class II (M5/114.15.2, #107639), anti-CD8 (53-6.7, #100742, #100750), anti-Granzyme B (GB11, # 515408), Cell signaling: anti-Calreticulin (D3E6, #19780), Thermofisher: anti-iNOS (CXNFT, #53-5920-82), anti-Ki-67 (SolA15, #11-5698-82), anti-TCRβ (H57-597, #45-5961-82), anti-CD44 (IM7, #56-0441-82), LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit, anti-TLR4 (UT41, #12-9041-80), CellROX™ Deep Red Flow Cytometry Assay Kit, FoxP3 Fixation and Permeabilization Buffer Set, and BD Bioscience: anti-active caspase-3 (C92-605, #559341), anti-CD45.1 (A20, #561872, #562452), anti-CD45.2 (104, #553772), anti-TNF (MP6-XT22, #554418, # 554419), anti-CD80 (16-10A1, #561954, #553769), anti-CD86 (GL1, #553691, #558703), anti-CD4 (RM4-5, #562285), 7-AAD, BD Cytofix/Cytoperm Plus Kit (with BD GolgiPlug), and antibody staining followed the manufacture's protocol. Cells were acquired using BD FACSCalibur or BD FACSCelesta, and data were analyzed using FlowJo (FlowJo, LLC).

RNA sequencing and data analysis

On day 14 after tumor inoculation, TAM ($CD45^+$ $7AAD^-$ $CD8^-$ $Ly6C^-$ $Ly6G^-$ $CD11b^+$ $F4/80^+$) from vehicle or JHU083 treated 4T1 tumor- bearing mice were sorted on BD FACSAria™ Fusion. For RNA sequencing analysis, total RNA (5 mice per group) was extracted using RNeasy Micro Kit (QIAGEN). Samples were sent to Admera Health for sequencing and analysis. $Poly(A)^+$ transcripts were isolated by NEBNext® Poly(A) mRNA Magnetic Isolation kit. Prior to first strand synthesis, samples were randomly primed and fragmented using NEBNext® Ultra™ RNA Library Prep Kit for Illumina®. The first strand was synthesized with Protoscript II Reverse Transcriptase. Samples were pooled and sequenced on a HiSeq with a read length configuration of 150 PE. The transcriptomic analysis work flow began with a thorough quality check by FastQC v0.11.2. The latest reference genome (GRCM38) were used for read mapping. The

statistical significant gene analysis in context of gene Ontology and other biological signatures were performed using Gene Set Enrichment Analysis (GSEA) and DAVID.

The Cancer Genome Atlas data analysis

Breast invasive carcinoma (N=1085) and normal (N=112) samples were analyzed and correlation plots were generated by using the GEPIA (Gene Expression Profiling Interactive Analysis, http://gepia2.cancer-pku.cn/#index) tool (73). Correlation plots were generated from these data focusing on major glutamine utilizing enzymes.

Generation of BMDMs

For the preparation of bone marrow cell suspensions, the bones of both hind limbs (two tibias and two femurs) were flushed with ice-cold DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 2 mM L-glutamine (cell media) plus 20% L929-conditioned media. The cells were incubated at 37 °C, and on day 4, non-adherent cells were removed, and cells were cultured with fresh L929 conditioned media. On day 7, BMDMs were lifted using Cellstripper (Mediatech, Manassas, VA). 1 x 10^6 cells BMDMs were seeded in 12-well plates, and treated with DON. To make tumor-conditioned media, tumor cells were cultured in the presence or absence of DON (0.5 μM or 1 μM). After 1 hour of incubation, cells were washed and replaced with fresh drug-free media. After 24 hours, supernatants were harvested and used as conditioned media. BMDMs were cultured in the presence of these conditioned media for 24 hours.

T cell priming assay by co-culture with OTI and tumor cells

3 x 10^5 BMDM cells were plated on a 12 well plate, then 50,000 B16 OVA or MC38 OVA cells were added. After attachment, different doses of DON were applied and incubated for 24 hours. $CD8^+$ T cells from OTI mice were isolated by negative $CD8^+$ T Cell Isolation Kit (Miltenyi Biotech). Purified OTI $CD8^+$ T cells from OT1 mice were labeled with Cell Proliferation Dye eFluor™ 450 (Invitrogen). After discarding

the supernatant, labeled 3 x 10^5 $CD8^+$ T cells or 2 x 10^6 whole splenocytes were co-cultured with BMDMs and tumor cells. After 72 hours, cells were analyzed by flow cytometry.

Immunoblotting

For nuclear and cytoplasm fractionation, 1 x 10^7 BMDMs were washed twice with PBS, then lysed cells in cytoplasm separation buffer composed of 10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% (v/v) NP40, 1 mM DTT, and 1 mM PMSF, and then lysed in a RIPA buffer with sodium fluoride, protease inhibitor, PMSF, sodium pyrophosphate, β-glycerophosphate and sodium vanadate. For whole lysate immunoblotting, cells were lysed in RIPA buffer with NaF, protease inhibitor, PMSF, sodium pyrophosphate, β-glycerophosphate and sodium vanadate for nuclear fractionation. Immunoblotting was performed using a standard protocol (Life Technologies). The following antibodies were used. Cell Signaling: anti-active caspase-3 (D175, #9661), anti-p-STAT3 (Tyr705) (D3A7, #9145) or (Ser727, # 9134), p-STAT1 (Ser727) (D3B7, #8826), anti-STAT1 (D1K9Y, #14994), anti-STAT3 (124H6, #9139), anti-NF-κB p65 (D14E12, #8242), anti-p-NF-κB p65 (Ser536) (93H1, #3033), anti-α-tubulin (DM1A, #3873), anti-C/EBPβ (LAP) (#3087), anti-IDO (D8W5E, #51851), anti-GAPDH (D16H11, #5174), and anti-β-actin (D6A8, #8457). Abcam: anti-LAMP2 (GL2A7, #ab13524). Santa Cruz: anti-LaminB (#sc-6216). Sigma-Aldrich: Pan-actin antibody (#A2066). All images were captured and analyzed using UVP BioSpectrum 500 Imaging System.

Enzyme-linked immunosorbent assay

Serum or cell culture supernatants were collected, and CSF3 (ThermoFisher and R&D Mouse G-CSF DuoSet ELISA), TNF, and IL-10 (ThermoFisher) were analyzed by ELISA as described by the manufacturer.

Metabolite Extractions

Tumor (95 mg) and lung (whole lung) were harvested on day 17 from 4T1 tumor-bearing mice treated with or without JHU083. To minimize ischemic time, the organs were harvested within 1 minute. Metabolites were extracted from tumor and lung in a methanol:water (80:20, v/v) extraction solution after homogenization with an ultrasonic processor (UP200St, Hielscher Ultrasound Technology). Samples were vortexed and stored at -80°C for at least 2 hours to precipitate the proteins. The metabolite containing supernatant was isolated after centrifugation at 15,000g for 10 minutes and dried under nitrogen gas for subsequent analysis by LC-MS.

Metabolite Measurement with LC-MS

Targeted metabolite analysis was performed with LC-MS. Dried samples were re-suspended in a 50% (v/v) acetonitrile solution and 4μL of each sample was injected and analyzed on a 5500 QTRAP triple quadrupole mass spectrometer (AB Sciex) coupled to a Prominence ultra-fast liquid chromatography (UFLC) system (Shimadzu). The instrument was operated in selected reaction monitoring (SRM) with positive and negative ion-switching mode as described. This targeted metabolomics method allows for analysis of over two hundreds of metabolites from a single 25min LC-MS acquisition with a 3ms dwell time and these analyzed metabolites cover all major metabolic pathways. The optimized MS parameters were: ESI voltage was +5,000V in positive ion mode and –4,500V in negative ion mode; dwell time was 3ms per SRM transition and the total cycle time was 1.57 seconds. Hydrophilic interaction chromatography (HILIC) separations were performed on a Shimadzu UFLC system using an amide column (Waters XBridge BEH Amide, 2.1 x 150 mm, 2.5μm). The LC parameters were as follows: column temperature, 40 °C; flow rate, 0.30 ml/min. Solvent A, Water with 0.1% formic acid; Solvent B, Acetonitrile with 0.1% formic acid; A non-linear gradient from 99% B to 45% B in 25 minutes with 5min of post-run time. Peak integration for each targeted metabolite in SRM transition was processed with MultiQuant software (v2.1, AB Sciex). The preprocessed data with integrated peak areas were exported from MultiQuant and re-imported into Metaboanalyst software for further data analysis (e.g. statistical analysis, fold change, principle components analysis, etc.).

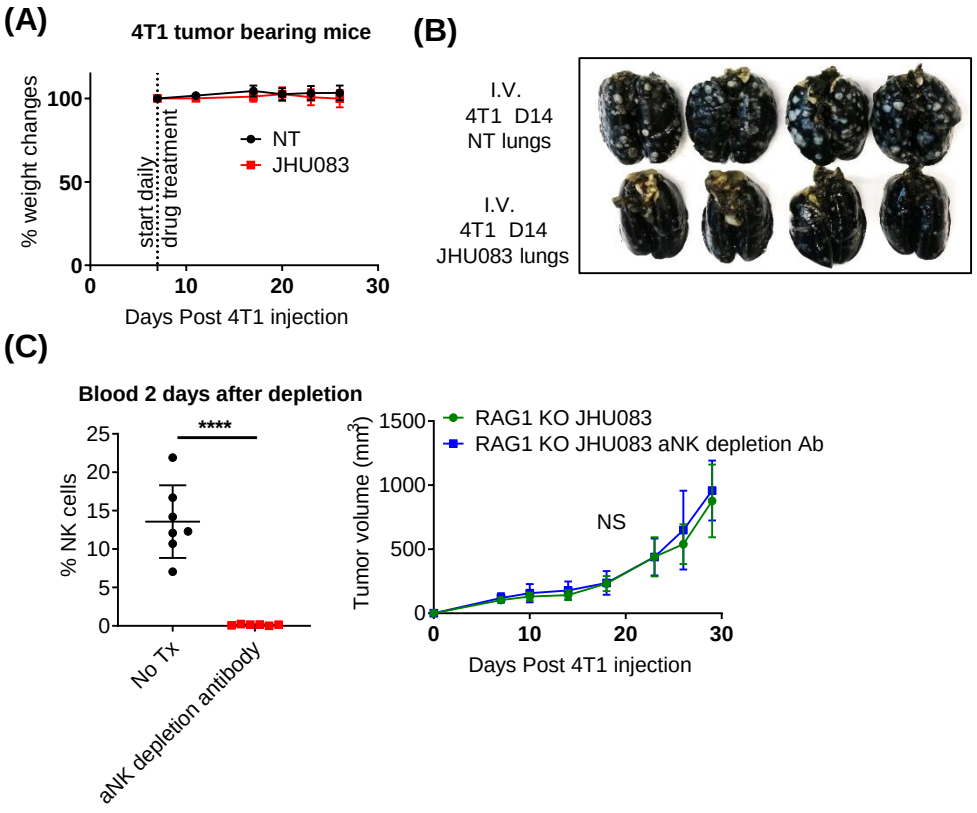
Immunohistochemistry

Lungs were inflated with 10% neutral buffered formalin by tracheal cannulation. Lungs were excised and placed in formalin for 24 hours. Formalin fixed lung section were paraffin-embedded and processed for histological analysis. Lung section were stained with hematoxylin and eosin (H&E), and images were captured and analyzed by microscope at 40 X magnification.

Lung metastasis analysis

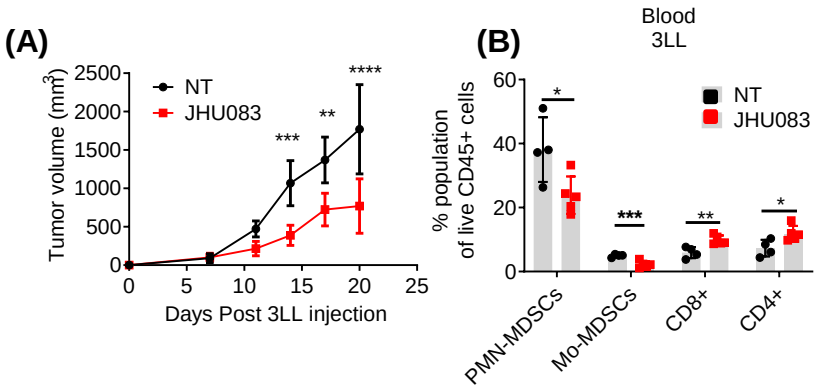
Lung metastases were analyzed by inflation with 15% india ink. After intra-tracheal injection of india ink, lungs were harvested and washed with in Feket's solution (70% ethanol, 3.7% paraformaldehyde, 0.75 M glacial acetic acid). Lungs were placed in fresh Feket's solution overnight, and surface white tumor nodules were counted in a group-blinded fashion using a Nikon stereomicroscope.

Supplementary Figure 1



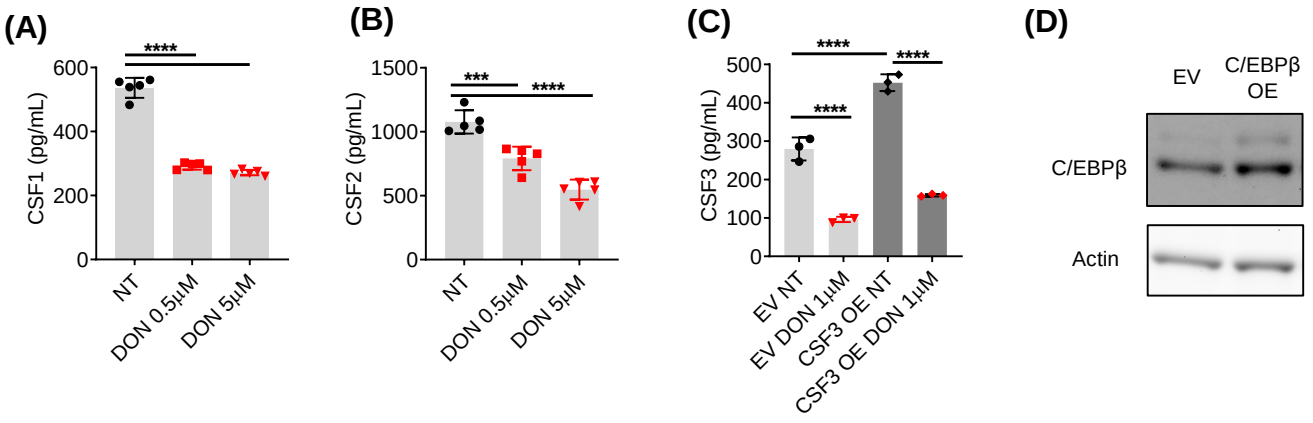
FigS1 related to Figure 1. Glutamine antagonism inhibits lung metastasis. (A) $1x10^5$ 4T1 cells were implanted subcutaneously into the mammary fat pad or (B) injected intravenously into the tail vein in BALB/cJ female mice. (A-B) 4T1 tumor-bearing mice were treated with JHU083 (1mg/kg) starting at day 7 after tumor inoculation. After 7 days of treatment, a lower dose (0.3 mg/kg) of JHU083 was used. (A) Mice weights were monitored and recorded. (B) On day 14, lungs were harvested, and lung metastasis were analyzed by inflation with 15% india ink to quantify tumor nodules. (C) RAG1 KO mice were treated intraperitoneally with anti-asialo GM1 depletion antibody for NK cell depletion on the day prior to tumor implant and then twice/week. ****P<0.001. Mann-Whitney tests (C, left). Two way ANOVA (C, right).

Supplementary Figure 2



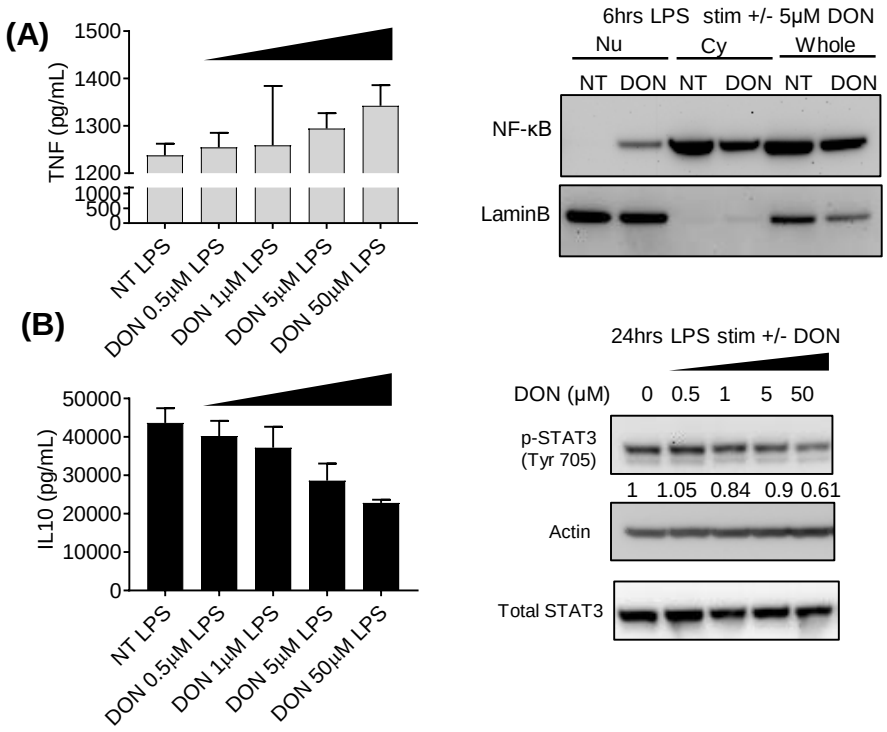
FigS2 related to Figure 2. Glutamine antagonism inhibits 3LL tumor growth, and reduces PMN-MDSCs and Mo-MDSCs in the blood. (A-B) $5x10^5$ 3LL cells were implanted subcutaneously into the right flank of C57BL/6J male mice. 3LL tumor-bearing mice were treated with JHU083 (1mg/kg) starting at day 7 after tumor inoculation. After 7 days of treatment, a lower dose (0.3 mg/kg) of JHU083 was used. (A) 3LL tumor sizes were measured. (B) Percentages of PMN-MDSCs, Mo-MDSCs, $CD8^+$, and $CD4^+$ of live cells from the blood in 3LL tumor bearing mice were analyzed by flow cytometry at day 15. *P < 0.05, **P < 0.01, ***P < 0.005, ****P<0.001. Two way ANOVA with bonferroni post test (A). Unpaired *t* test (B).

Supplementary Figure 3



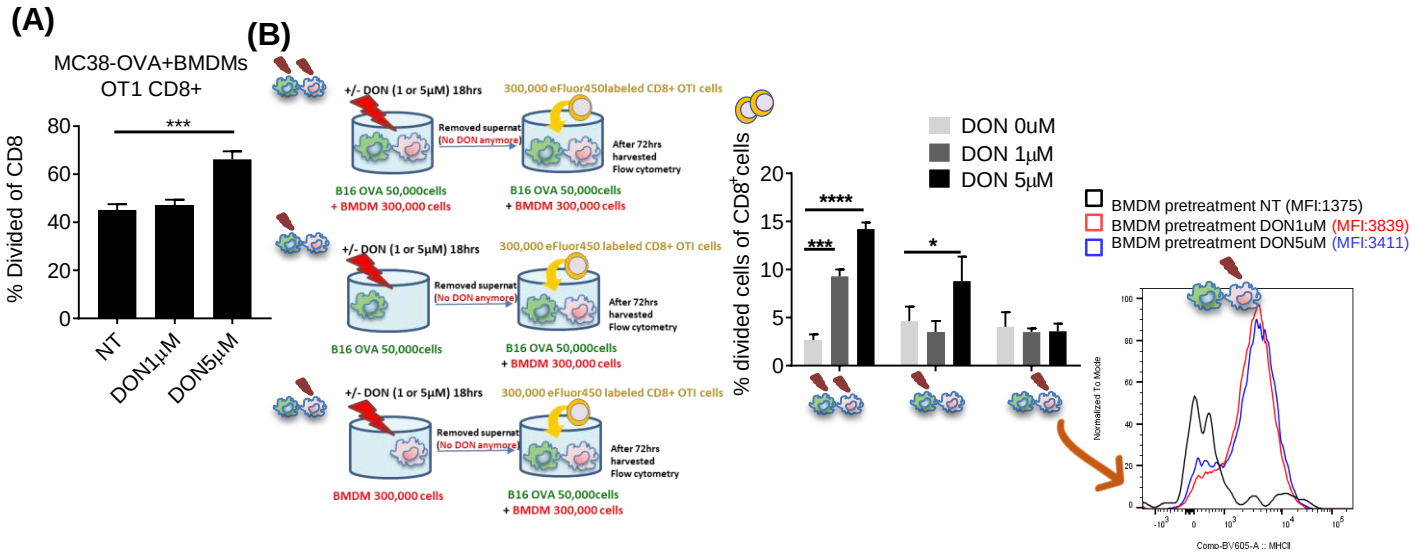
FigS3 related to Figure 3. Glutamine antagonism inhibits CSF1/ 2/ 3 secretion. (A and B) 4T1 cells or (C) EV (empty vector) or CSF3 OE (overexpressing) 4T1 cells were cultured in the presence or absence of DON (0.5 μM, or 5 μM) for 24 hours. (A) CSF1, (B) CSF2, or (C) CSF3 levels were measured by ELISA. (D) C/EBPβ expression level in in vitro cell lysates was measured on EV or C/EBPβ OE 4T1 cells. ***P < 0.005, ****P <0.001. One way ANOVA with Tukey post test (A-C).

Supplementary Figure 4



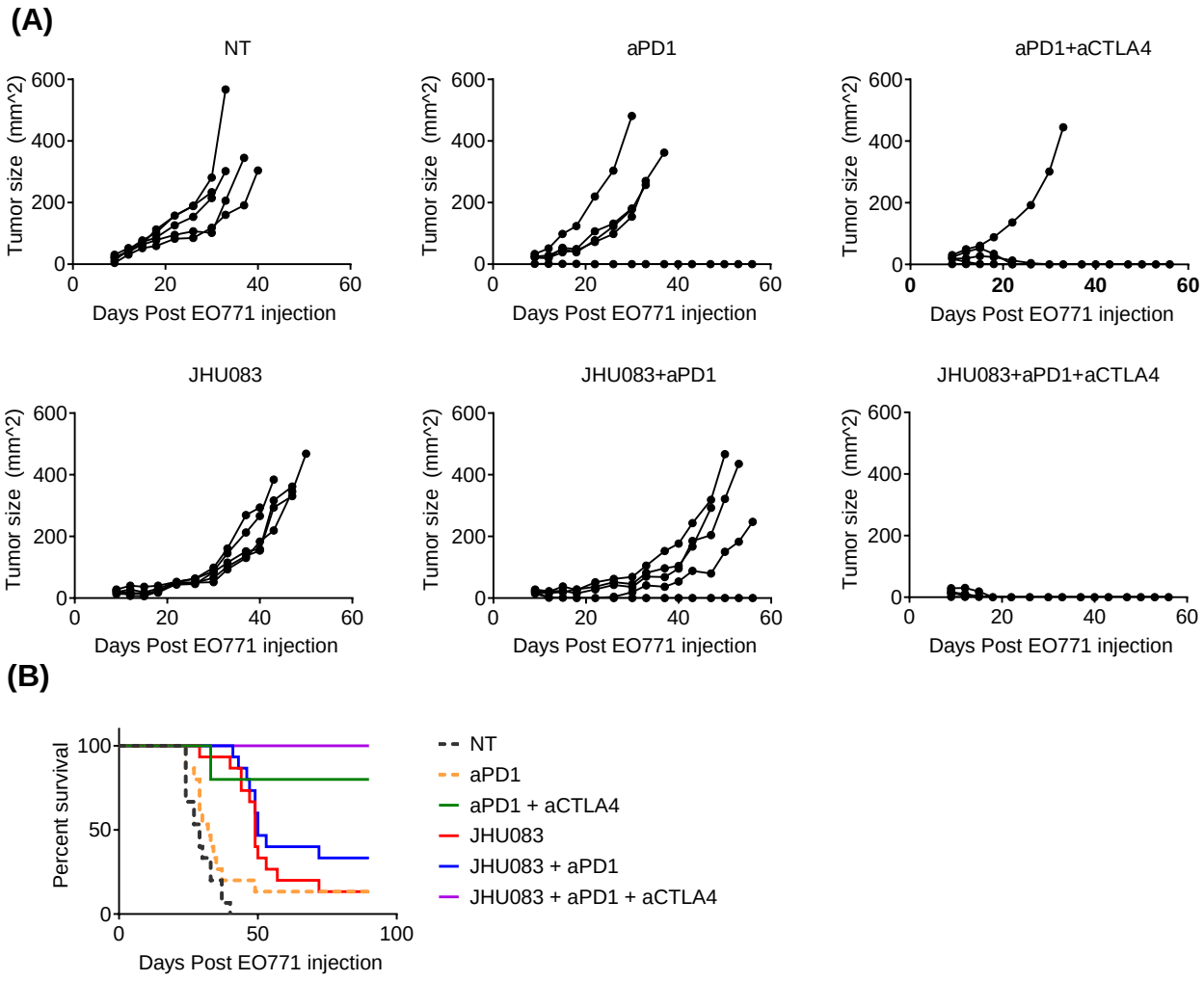
FigS4 related to Figure 4. Glutamine antagonism enhances proinflammatory macrophage phenotypes. (A) BMDMs were stimulated with LPS in the presence or absence of indicated dose of DON for 6 or 24 hours. After 24 hours, supernatants were collected and TNF levels were measured by ELISA (left). BMDMs were stimulated with LPS and treated with or without DON. NF-κB levels were probed in nuclear (Nu) and cytosolic (Cy) fraction from LPS or LPS+5μM DON treated (6 hours) BMDMs by immunoblotting. LaminB was used to confirm the nuclear fraction (right). (B) IL-10 in supernatants were measured by ELISA (left). p-STAT3 (Tyr705) level, actin, and total STAT3 from BMDMs stimulated with either LPS, LPS+DON, or LPS for 24 hours were measured by immunoblotting (right).

Supplementary Figure 5



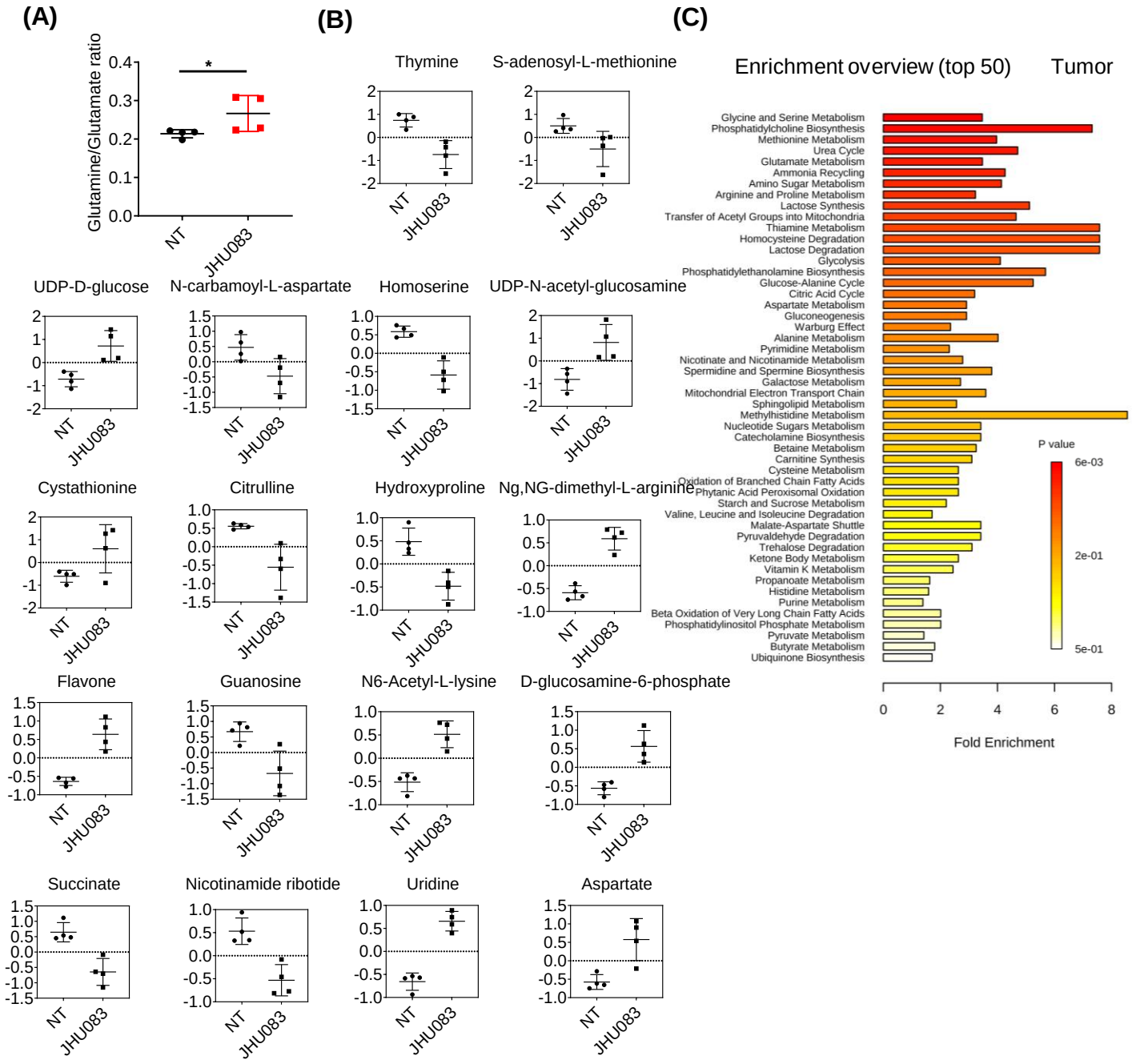
FigS5 related to Figure 6. Glutamine antagonist treated BMDMs enhance antigen presentation (A) $3x10^5$ BMDMs and $5x10^4$ MC38-OVA tumor cells were co-cultured in the presence or absence of 1 µM or 5 µM of DON. After 24 hours of incubation, supernatants were discarded and $2x10^6$ eFluor450-labeled whole splenocytes from OTI mice were added. Percentages of divided cells from $CD8^+$ population were analyzed by flow cytometry. (B) BMDMs or B16-OVA tumor cells were cultured in the presence or absence of 1 µM or 5 µM of DON. After 18 hours of incubation, $3x10^5$ BMDMs or $5x10^4$ B16-OVA tumor cells were seeded and $3x10^5$ eFluor450-labeled $CD8^+$ T cells from OTI mice were added. Scheme of the experiment (Left) Percentages of divided cells from $CD8^+$ population were analyzed by flow cytometry (middle). Histogram showing MHCII expression (right). One way ANOVA with bonferroni post test (A). Two way ANOVA with Tukey post test (B).

Supplementary Figure 6



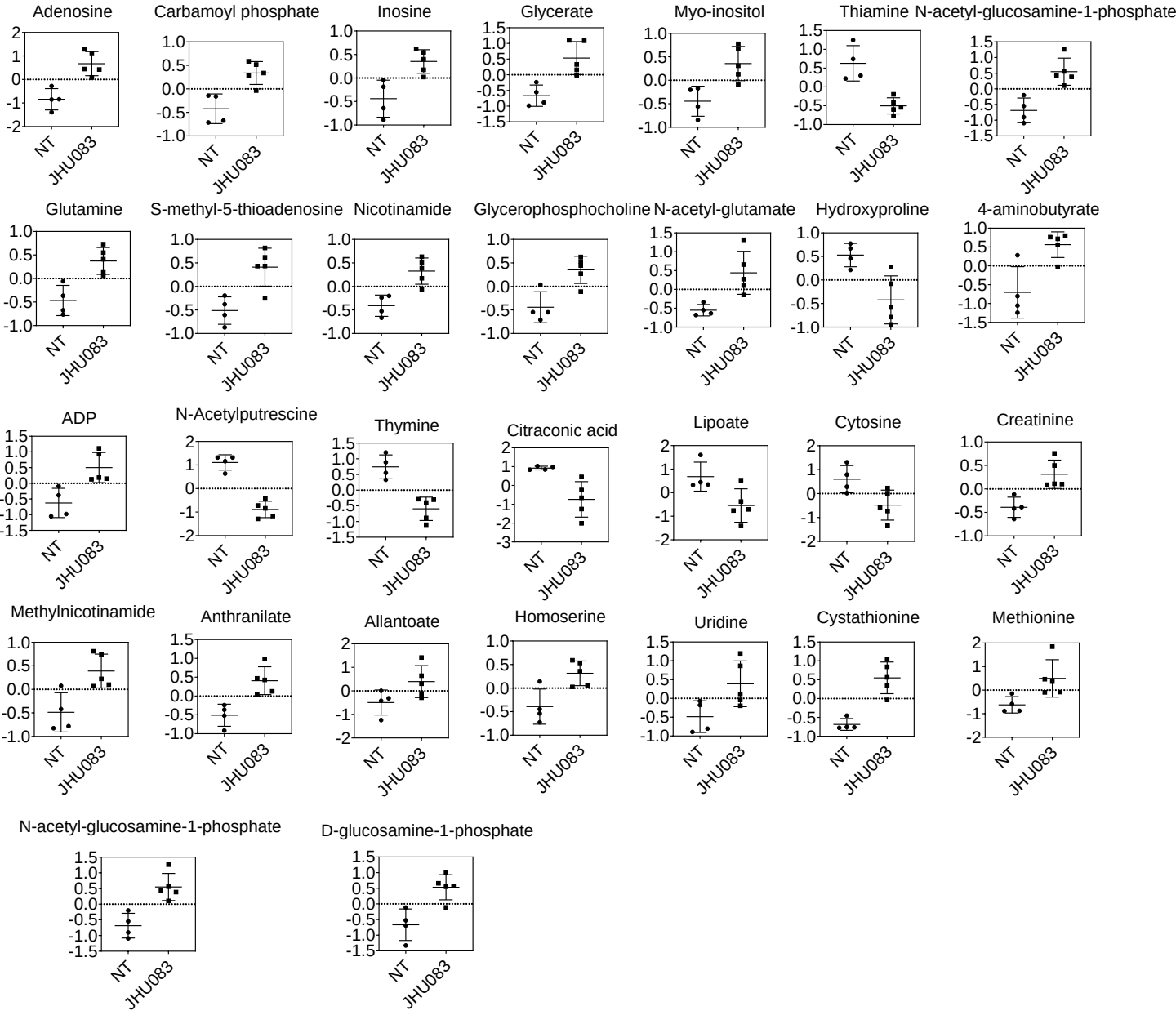
FigS6 related to Figure 8. Glutamine antagonism enhances immune checkpoint blockade in EO771 tumor-bearing mice. 2x10^5 EO771 cells were implanted subcutaneously into the mammary fat pad of C57BL/6J female mice. EO771 tumor-bearing mice were treated with JHU083 (1 mg/kg) daily starting at day 7 after tumor inoculation. After 7 days of treatment, JHU083 daily dose was reduced (0.3 mg/kg). On days 9, 12, and 15, indicated mice were injected with 100 µg anti-PD1 or 100 µg anti-CTLA4 followed by treatment with or without JHU083. (A) Each individual mice tumor growth curve and (B) survival curve were recorded (N=5/group). Data are representative of at least three independent experiments.

Supplementary Figure 7



FigS7 related to Figure 9. Summary of metabolic changes of tumors from vehicle vs. JHU083 treated 4T1 tumor bearing mice. 1x10^5 4T1 cells were implanted subcutaneously into the mammary fat pad of Balb/cJ female mice. 4T1 tumor-bearing mice were treated with JHU083 (1mg/kg) starting at day 7 after tumor inoculation. After 7 days of treatment, On day 14, the tumors were harvested. (A) Graphs of ratio of glutamine and glutamate. (B) Significant metabolites from 4T1 tumors (related to Figure 9A-C) (C) Pathway analysis from significantly change metabolites in tumor. *P < 0.05, Mann-Whitney *t* tests (A).

Supplementary Figure 8



FigS8 related to Figure 10. Summary of metabolic changes of lungs from vehicle vs. JHU083 treated 4T1 tumor bearing mice. 1x10^5 4T1 cells were implanted subcutaneously into the mammary fat pad of Balb/cJ female mice. 4T1 tumor-bearing mice were treated with JHU083 (1mg/kg) starting at day 7 after tumor inoculation. After 7 days of treatment, On day 14, the lungs were harvested. Significant metabolites from lungs of 4T1 tumor bearing mice (related to Figure 10A-C). *P < 0.05, Mann-Whitney tests.