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Supplementary Information for

JAK/STAT inhibition in macrophages promotes therapeutic resistance by inducing expression of pro-tumorigenic factors

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Supplementary text
Figs. S1 to S7

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Supplementary Information Text
Materials and Methods.

Cell Culture
4T1 cells were grown in media containing RPMI, 10% FBS, 1% penicillin/streptomycin (Life Technologies), 1% L-glutamine (Life Technologies), 10 mM HEPES (Life Technologies), 1 mM sodium pyruvate (Life Technologies) 200 µg/mL G418. MDA-MB-231, Hs578T, MDA-MB-468, and BT-474 cells were grown in media containing DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. MCF7, ZR-75-1, T47D, SKBR3, and BT-549 cells were all grown in their respective culture conditions recommended by ATCC. MCF-10A cells were grown in DMEM/F-12 (Lonza) supplemented with 5% horse serum and additionally supplemented with 1mg insulin, 10µg EGF, 0.02% cholera toxin, 0.005% hydrocortisone and 1% penicillin/streptomycin. THP-1 monocytes were maintained in media containing RPMI supplemented with 10% FBS and 1% penicillin/streptomycin. Human primary macrophages were derived from PBMCs isolated from Trima Cones obtained through the Memorial Blood Center. PBMCs were subjected to CD14+ enrichment via MACS LS columns and differentiated into macrophages as described below. All cells were grown at 37°C and 5% CO_2 and regularly checked for mycoplasma contamination.

Cell stimulation
Serum-starved HC-11/R1 cells were treated with 30 nM B/B (Clontech) or vehicle (ethanol) for 24 h, and conditioned medium (B/B CM) was collected, filtered, and used to stimulate BMDMs. THP-1 cells were differentiated into macrophages with 5 ng/mL phorbol 12-myristate 13-acetate (PMA) overnight. Conditioned medium (CM) collected from serum-starved breast cancer cell lines was spun down to eliminate cellular debris and used to stimulate differentiated THP-1 or primary human macrophages that were serum-depleted in 1% FBS in DMEM for 4 h prior to CM exposure. THP-1 or primary macrophages used for in vitro studies with ruxolitinib were simultaneously treated with 0.5µM or 10µM ruxolitinib or DMSO during the 4 h pre-stimulation period as described above, then exposed to tumor CM and drug or control. Protein was collected for immunoblot analysis or after 24 h of incubation, media was replaced with fresh serum-free media for an additional 24 h to collect the soluble factors from the JAK-inhibited TAMs. This double-conditioned media, referred to as either Rux (ruxolitinib) or DMSO TAM CM was used to make dilutions with ruxolitinib and introduced to tumor cells plated in 96-well plates at 1x105 cells/0.1 mL in 1% FBS DMEM to assess the half maximal inhibitory concentration (IC50) of ruxolitinib. Dilutions of Rux for the IC50 assay were made in DMSO TAM CM or Rux TAM CM. Studies with celecoxib in THP-1 or primary macrophages were carried out as described above with the addition of 10µM celecoxib-only and celecoxib plus ruxolitinib treatment groups. PBMCs were subjected to CD14+ enrichment via CD14+ microbeads (Miltenyi Biotec) through MACS LS columns (Miltenyi Biotec) and differentiated into macrophages with recombinant M-CSF (BioLegend) in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin on days 1 and 5 (Day 5 treatment with 2X M-CSF) following CD14+ enrichment. In experiments blocking the IL-6 receptor, gp130, with SC-144 (Sigma), THP-1 macrophages were pre-treated with 50µM SC-144 or control during the 4 h incubation with 1% FBS prior to breast cancer CM exposure.

ELISA
At 24 h, conditioned media was collected from serum starved MCF10A, Hs578T, MDA-MB-231, MDA-MB-468, BT549 cells. ELISAs for human IL-6 were performed according to the manufacturer's protocol (R&D Systems). Conditioned media samples collected for PGE2

ELISAs were first concentrated using Amicon Ultra-15 centrifugal filter units (EMD Millipore), then performed according to the manufacturer's protocol (Enzo).

Proteome Profiler Mouse XL Cytokine Array
HC-11/R1 and 4T1 cells were grown to confluence and serum-starved for 24 h. Medium was changed and HC-11/R1 cells were treated with 30 nM B/B homodimerizer or solvent control for 24 h. 4T1 cells were incubated with serum free medium for 24 hours. The supernatant was collected, centrifuged to remove precipitation and cell debris and concentrated using Amicon Ultra-15 centrifugal filter units (EMD Millipore). Cytokines and chemokines in the supernatant were measured according to the protocol for the Proteome Profiler Mouse XL Cytokine Array (R&D Systems). Data were obtained and analyzed using UN-SCAN-IT gel analysis software (Silk Scientific). Representative data are shown in bar graphs.

Bio-Plex™ Array
Breast cancer cell lines were plated in 6 cm dishes and allowed to grow to 70-80% confluency. Cells were then serum-starved for 24 h and conditioned medium samples were collected and spun at 1000 xg for 15 min at 4˚C. BSA in PBS was then added to each sample for a final concentration of 0.5% to stabilize protein analytes and prevent absorption into labware. Each sample was then transferred to a clean eppendorf tube and spun at 10,000 xg for 10 min at 4˚C, after which the supernatants were used to perform the assay according to the manufacturer's instructions (BioRad).

Analysis of Human Breast Cancer Samples
21 cases each of TNBC, $HER2^+$, and $ER^+/HER2^-$ breast cancer were selected matched for Nottingham grade, tumor size, and proportion of lymph node positive disease. Each case was represented by quadruplicate cores on the TMA. Co-positive cells were defined by 3+ cytoplasmic and/or membrane staining for CD68 with 3+ nuclear staining for pSTAT3.

***In vivo* treatments**
Ruxolitinib was originally resuspended in DMSO and diluted in 1% Tween-20/PBS for gavage. Treatments consisted of daily oral gavage of 60 mg/kg ruxolitinib or DMSO (vehicle) and 0.8 mg/mouse injection via i.p. of control or clodronate liposomes (Encapsula Nano Sciences) followed by 0.4 mg/mouse liposome injection via i.p. every 4 days. Treatments consisted of a daily oral gavage of 60 mg/kg ruxolitinib or equivalent volume of DMSO and a second daily oral gavage of 75 mg/kg Celecoxib (Pfizer), resuspended in DMSO and diluted in PEG-400 and ddH_2O for gavage, or equivalent DMSO, depending on the treatment group. $STAT3^{cKO}$ mice were given either DMSO (control) or 75 mg/kg Celecoxib via daily oral gavage once 4T1 mouse tumors reached $200mm^3$. Tumor sizes were measured using calipers. All mice were injected with 30 mg/kg 5-bromo-2'-deoxyuridine (BrdU, supplied by Sigma) i.p. 2 h prior to sacrifice.

Flow cytometry
Tumors were harvested by blunt dissection and single-cell suspensions were made by mechanical disruption at room temperature, followed by 30 min incubation at 37˚C in 24µg/ml Liberase TL (Roche, #05401020001) and 0.15mg/ml DNase I (Sigma-Aldrich, #DN-25). Digestion was halted by addition of DMEM with 10% FBS and centrifugation, followed by passing the cell suspension through a 70µm strainer (Falcon, #352350). Red blood cells were lysed using ACK Lysing Buffer (Lonza, #10-548E) following manufacturer's instructions. Following addition of ice-cold PBS, cells were centrifuged at 4˚C for 5 min at 65 xg, and remaining cells were resuspended in pre-chilled FACS buffer (0.5% FBS, 10mM EDTA, 1X PBS). Samples were stained with antibodies listed below and analyzed on an LSRII (Becton Dickinson). Fixable Viability Dye eFluor 780

was added to all samples to exclude dead cells (eBioscience, #65-0865-14). Cells were resuspended in Foxp3 transcription factor fix/permeabilization reagent (eBioscience00-5523-00) overnight and stained with Foxp3-AF647 (BioLegend 126408). T regulator cells (Tregs) were defined as CD3-positive CD25-positive FoxP3-positive. All flow data were analyzed in FlowJo software (Tree Star v.10).

Antibodies
For flow cytometry: Purified CD16/32 (eBioscience, #14-0161), F4/80-FITC (BioLegend, #123107), CD45-Pe/Cy7 (BioLegend 103113), CD45-AF700 (BioLegend 103128), CD3-BV785 (BioLegend 100232), CD3-APC (BioLegend 100311) CD4-PerCP/Cy5.5 (BioLegend 100434), CD8-BV510 (BioLegend 100752), CD11b (BioLegend 101222), CD11c (BioLegend 117347), CD25-Pe/Cy7 (BioLegend 101916), CD44-FITC (BioLegend 103006), Ly6G (BioLegend 127615), Ly6C (BioLegend 128033). For immunoblot analysis: pSTAT3 (Cell Signaling #9131, 1:1000), STAT3 (Cell Signaling #12460, 1:1000), pSTAT5 (Cell Signaling #9359, and total STAT5 (Cell Signaling #9363) and GAPDH (Cell Signaling #2118, 1:10000).

Immunostaining
Frozen sections were permeabilized for 10 minutes at -20˚C in pre-chilled methanol. Following an hour block in 10% normal goat serum, sections were stained for F4/80 (1:100, Abd Serotec #MCA497GA) at 4˚C overnight. Secondary antibody goat anti-rat Alexa Fluor 568 were incubated for 1 h at room temperature (1:400, Invitrogen, #A11007), and tissues were coverslipped with ProLong Gold Antifade DAPI (Invitrogen, #P36931). For analysis of paraffin embedded sections, tumors were fixed in 4% paraformaldehyde and paraffin embedded. 5µm thick sections were stained with hematoxylin and eosin (H&E), F4/80 (1:100, no antigen retrieval, BioRad, #MCA49RT) and BrdU (1:200, Abcam, #ab6326) as previously described. Cell death was immunostained using DeadEnd Fluorometric TUNEL System (Promega #9FB055). (1:100, antigen retrieval, BioRad, #MCA1815T) and pSTAT3 (1:100, antigen retrieval, Cell Signaling Technology, #9145). Human sections were stained with CD68 (1:100, antigen retrieval, BioRad, #MCA1815T) and pSTAT3 (1:100, antigen retrieval, Cell Signaling Technology, #9145).

Microscope Image Acquisition
All images were taken on a Leica DM400B microscope at either 20x or 40x objectives. Images were acquired using a Leica DFC310 FX camera and LAS V3.8 software and processed in Photoshop. 5 images of at least 3 representative tumors were analyzed. Images for the human samples were acquired on (Nikon NiE C2 Upright Confocal Microscope, 40x, University Imaging Center, University of Minnesota).

Primer Sequences
IL6: Fwd- TAG CCG CCC CAC ACA GAC AG, Rev- GGC TGG CAT TTG TGG TTG GG
GMCSF: Fwd- CGT CTC CTG AAC CTG AGT AGA, Rev- TGC TGC TTG TAG TGG CTG G
PTGS2: Fwd- GCC TGG GGT GAT GAG CAG TT, Rev- CAG AAG GGC AGG ATA CAG C
EREG: Fwd- ATG TGG CTT TGA CCG TGA TTC, Rev- TCC CCT GAG GTA ACT CTC TCA TA
THBS1: CCA GCT GTA CAT CGA CTG TGA, Rev- GCA GAT GGT AAC TGA GTT CTG A
JAG1: Fwd- TGC CAA GTG CCA GGA AGT, Rev- GCC CCA TCT GGT ATC ACA CT.

RNA-seq analysis
Strand-specific RNA-seq libraries were created using TruSeq Stranded mRNA Library Prep Kit (Illumina) and quality control was performed using PicoGreen Quantification and Agilent 2200

TapeStation sizing. Samples were multiplexed in one lane of an Illumina HiSeq 2500 and 50bp paired-end reads were sequenced (mean of 12.5 million pairs per sample). To pass quality control, at least 500 ng of RNA determined by RiboGreen Quantification and an RNA integrity number (RIN) of at least 8.0 as determined by Agilent 2200 TapeStation was required. Quality control on raw sequence data for each sample was performed with FastQC (v 0.11.5). Read mapping was performed via Hisat2 (v 2.0.2-beta) using the human reference genome (Ensembl GRCh38 release 89) or mouse reference genome (Ensembl GRCm38 release 94) when appropriate. Gene level quantification was completed using Subread featureCounts (v1.4.6) and principal components analysis (PCA) was completed in R on the 500 most variable genes using the "prcomp" function with rlog transformed data. PCA revealed a clear outlier (231CM.RUX.1) that had severe sequencing problems (only 2 million reads mapped to genes and the most highly expressed genes were a group of RNA genes, suggesting that mRNA enrichment during library prep failed in this sample). This sequencing outlier was removed from any further analysis. Individual gene expression plots (e.g. PTGES) were created by calculating transcripts per million mapped reads (TPM) values in each sample. Differentially expressed genes were identified using DESeq2 (negative binomial model v1.16.1). Group comparison p-values were adjusted by the Benjamini and Hochberg method to account for multiple hypothesis testing where genes with a False Discovery Rate (FDR) $q < 0.05$ were investigated in downstream analyses. For assessment using the MTCI Breast Cancer Survival Analysis Tool, within each individual breast cancer dataset, a gene's expression distribution was determined and patient tumors were classified as expressing high or low levels of the gene, if their expression was in the upper or lower quartile (>75%, or <25%, respectively). Patients with tumors classified as either high or low expressers were then combined across datasets for Kaplan Meier survival analysis.

Gene Set Enrichment Analysis (GSEA)

GSEA v3.0 (Broad Institute) was performed using pre-ranked gene lists derived from the DESeq2 differential expression values (log2 fold change and p-value). For each gene in a two-sample comparison, the fold change sign (+/-) was multiplied by the corresponding -log10(p-value) and sorted (from significantly expressed in one sample to significantly expressed in the other sample). Gene set enrichment statistics were calculated independently for each gene set collection in the Molecular Signatures Database (MSigDB, v 6.1). Interesting gene sets with FDR $q < 0.05$ were investigated further. Analyses were performed with 1000 permutations, classic scoring scheme, and other default parameters.

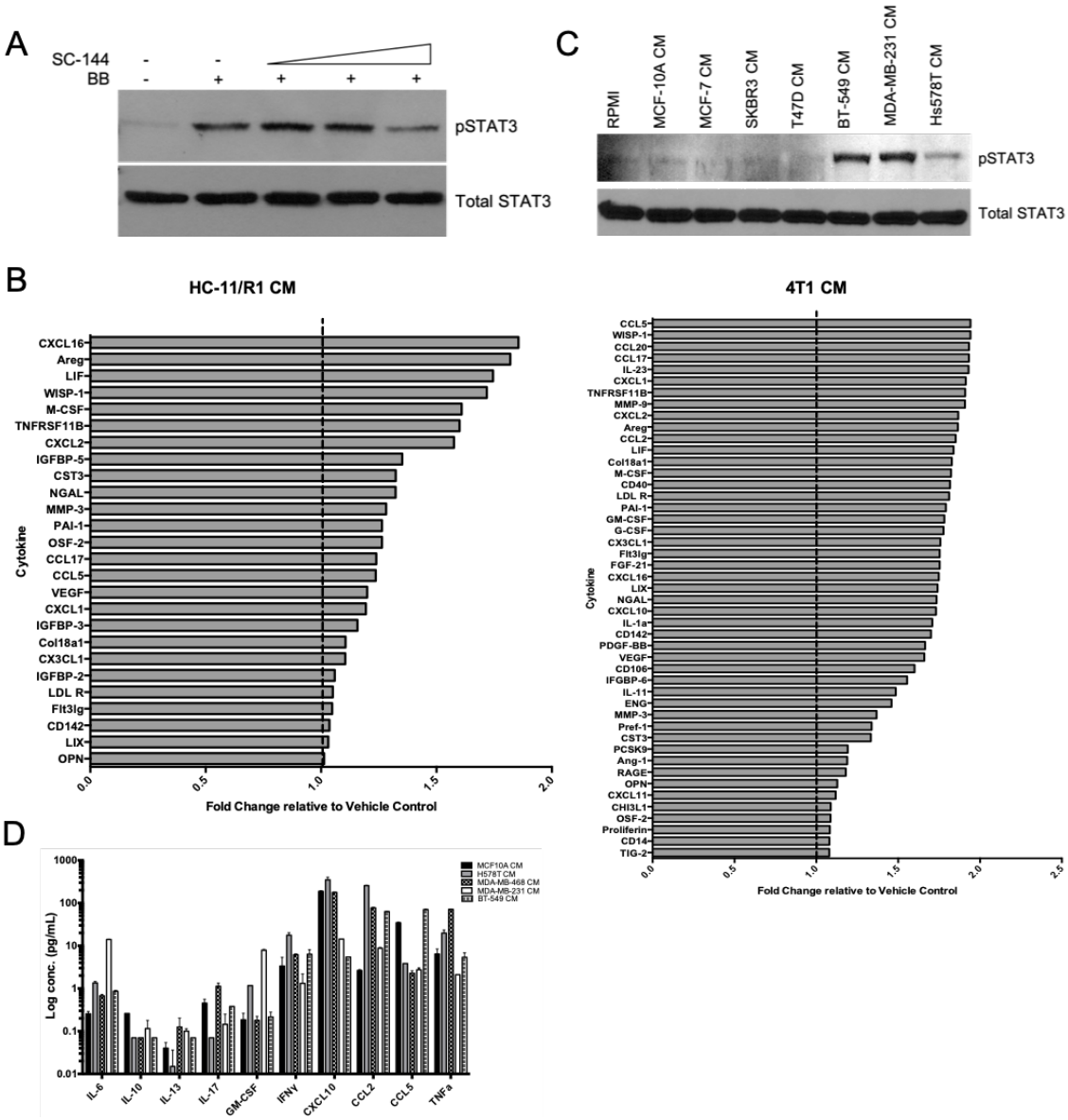


Fig. S1. IL-6 family members among others are important for STAT3 activation in macrophages. A. HC-11/R1 cells were treated with B/B (+) or solvent control (-) for 24 hours, conditioned medium was collected and used to stimulate BMDMs for 30 mins with SC-144 at various concentrations. B. Antibody array against 111 proteins performed on HC-11/R1 CM (left) and 4T1 CM (right) normalized to vehicle control media (dotted line). Spot pixel density subtracted from the averaged background signal representing the densitometry of spotted antibody array results. C. Immunoblot analysis for pSTAT3 and total STAT3 protein in primary PBMC-derived macrophages treated with breast cancer cell line CM for 15 minutes. D. Bioplex array results of STAT-activating cytokines in the CM collected from TNBC cells lines: Hs578T, MDA-MB-468, MDA-MB-231, and BT-549 cells normalized to CM from MCF-10A cells.

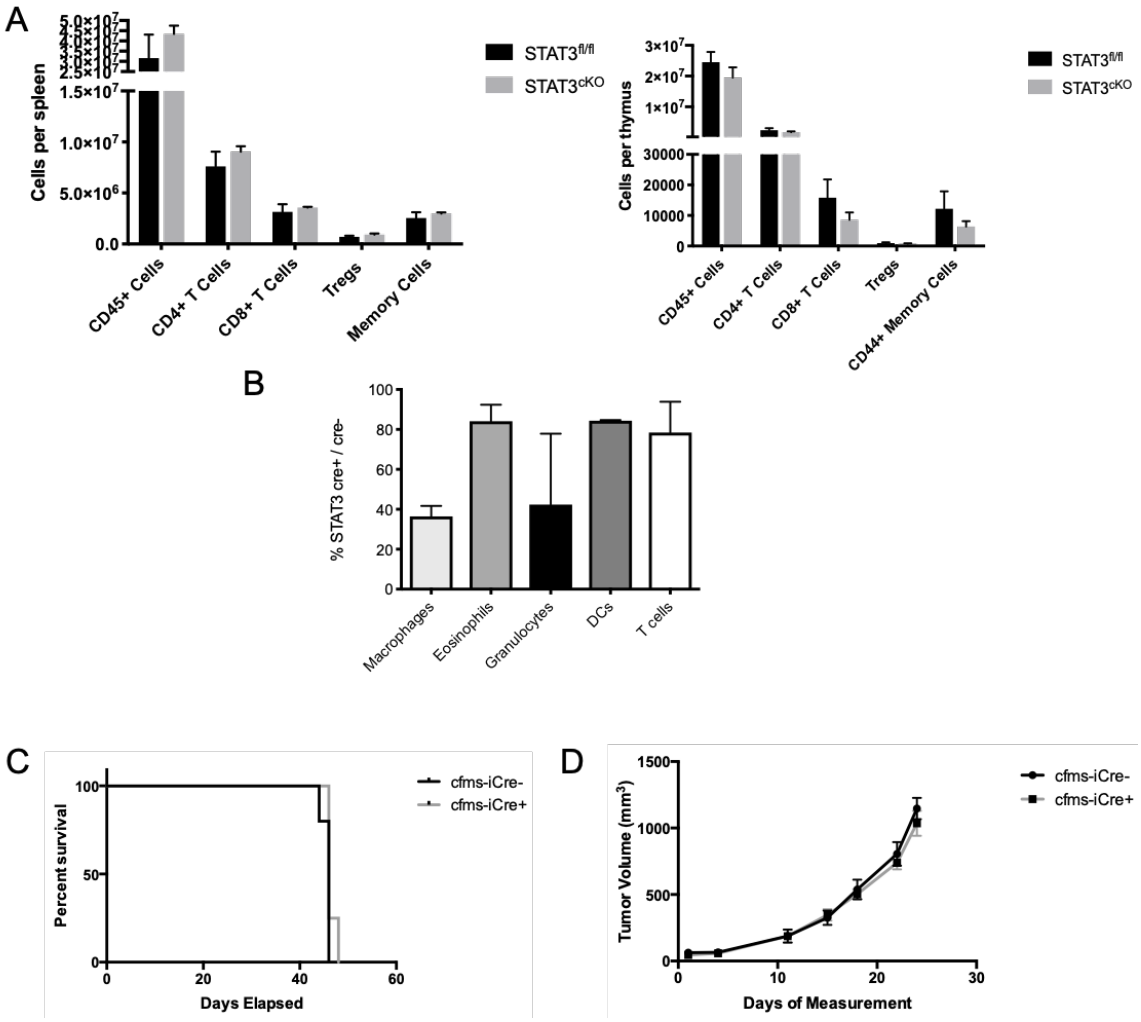


Fig. S2. Immune cell characterization of non-tumor-bearing conditional deletion mice. A. Immune cell characterization of non-tumor Balb/C mice via flow cytometry. Total cell counts were measured per organ. No significant differences between $STAT3^{fl/fl}$ and $STAT3^{cKO}$ mice. B. Immune cell characterization of HC-11/R1 tumors from $STAT3^{fl/fl}$ and $STAT3^{cKO}$ mice via flow cytometry. Percentage of Total STAT3-positive cells in $STAT3^{cKO}$ mice compared to $STAT3^{fl/fl}$ mice per cell type. Various immune cell types were labeled. C. Kaplan-Meier curves and D. Tumor growth curves of 4T1-Luc cells transplanted into mammary fat pads of 6-week-old iCre+ (n=4) or iCre- (n=5) Balb/c mice.

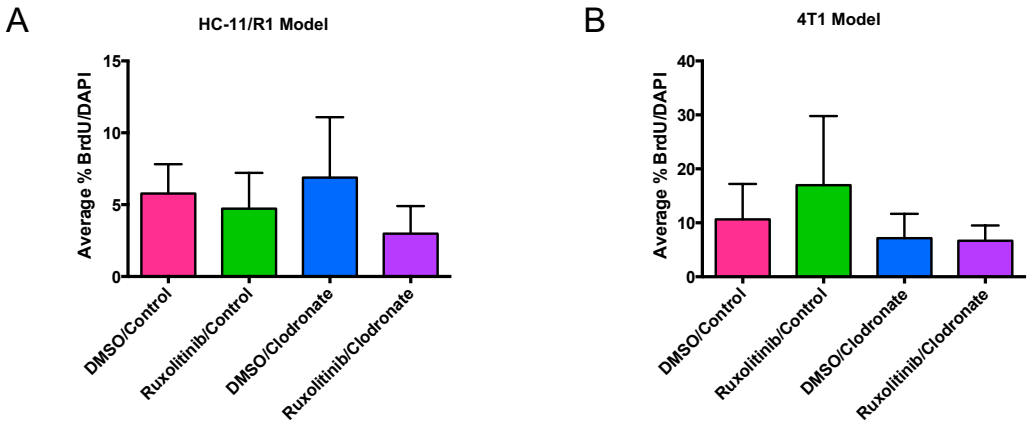


Fig. S3. Proliferation in tumors among ruxolitinib and clodronate treated mice. A. Quantification of percent of BrdU-positive cells relative to DAPI-stained nuclei in representative images in HC-11/R1 experiments. B. Quantification of percent of BrdU-positive cells relative to DAPI-stained nuclei in representative images in 4T1-Luc experiments.

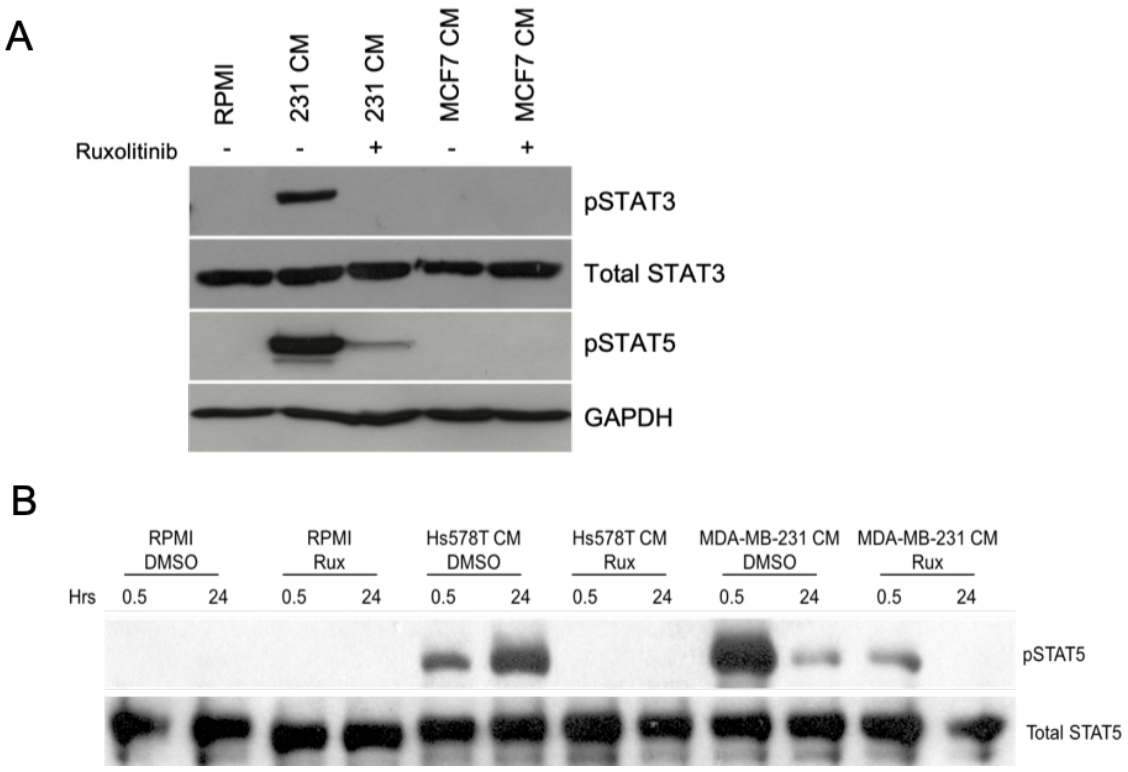


Fig. S4. Ruxolitinib inhibits STAT activation in macrophages A. Immunoblot analysis for pSTAT3, total STAT3, pSTAT5, and total STAT5 protein in primary PBMC-derived macrophages treated with RPMI, MDA-MB-231 CM, or MCF7 CM (negative control) with or without ruxolitinib for 30 min. B. Immunoblot for pSTAT5 and total STAT5 of THP-1 macrophages treated with RPMI, Hs578T, or MDA-MB-231 CM with or without Rux for 30 min and 24h.

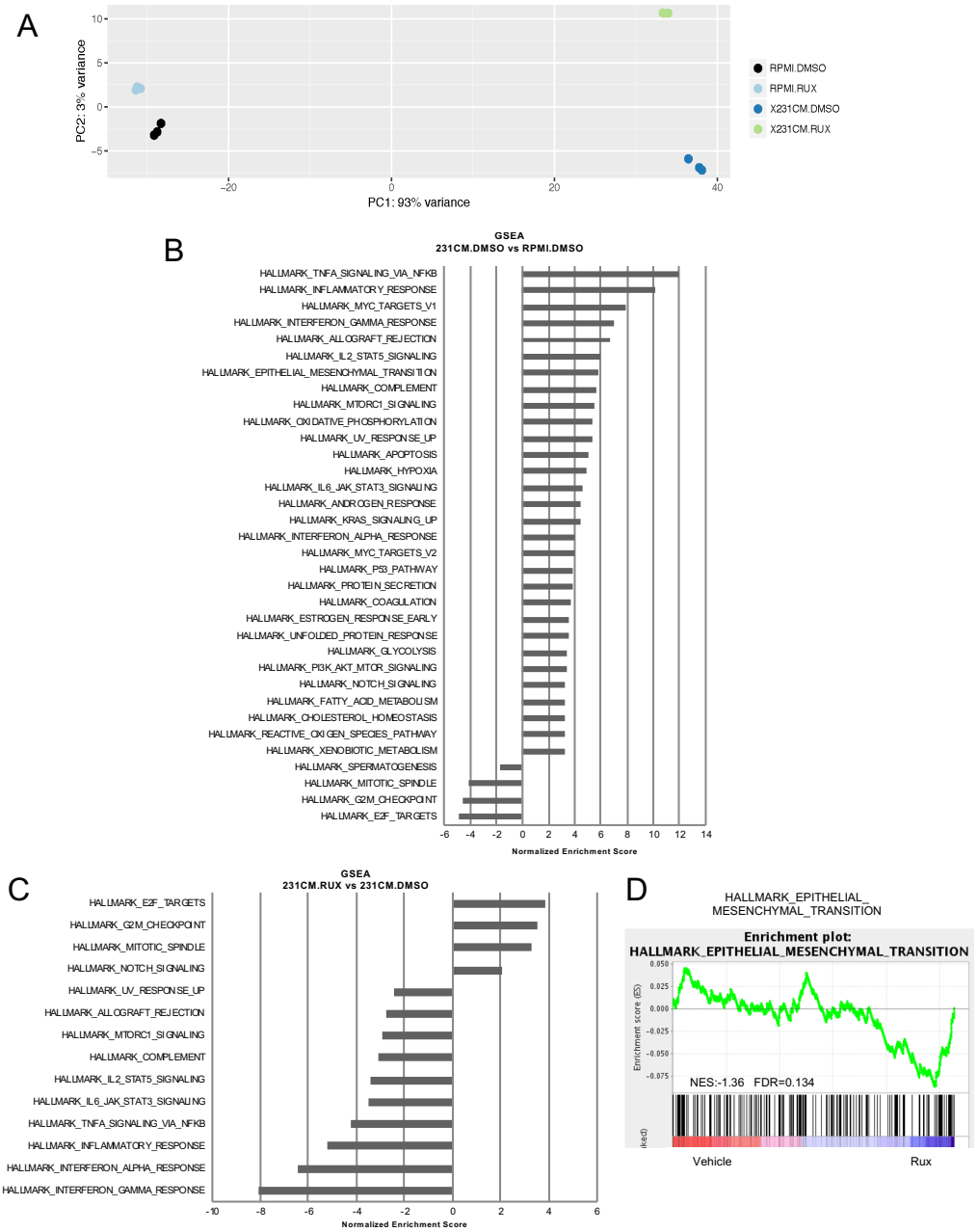
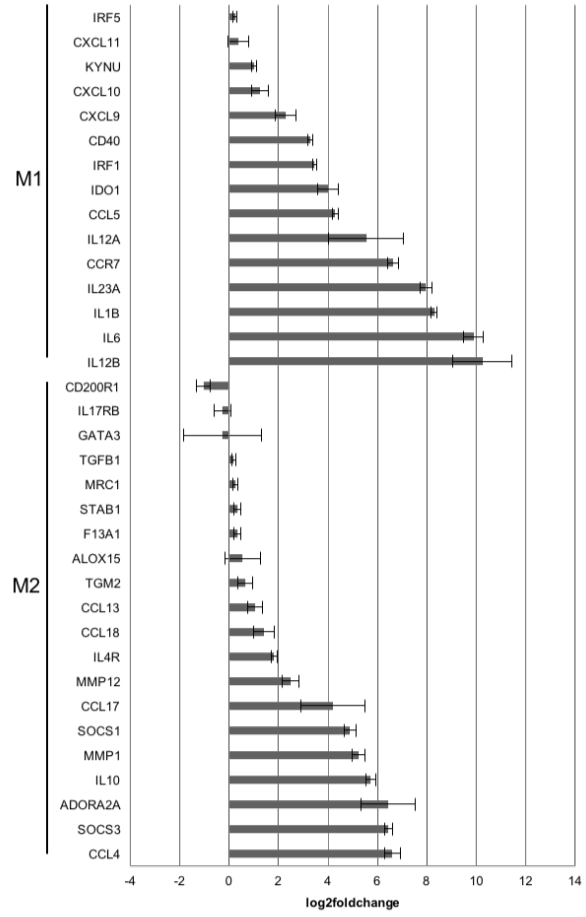


Fig. S5. JAK inhibition in macrophages induces expression of genes associated with cancer-related pathways. A. PCA analysis of samples treated with ruxolitinib in the presence or absence of conditioned media from MDA-MB-231 cells. B. GSEA demonstrating pathways altered in the PBMC-derived macrophages treated with tumor cell conditioned medium compared with control (RPMI) medium, sorted by NES, all with FDR < 0.05. C. GSEA demonstrating pathways altered in the PBMC-derived macrophages treated with tumor cell conditioned medium and ruxolitinib compared with tumor cell conditioned medium and DMSO (solvent control), sorted by NES, all with FDR < 0.05. D. GSEA analysis of an EMT pathway in PBMC-derived macrophages treated with CM in the presence of vehicle of ruxolitinib.

A



B

Gene	Log2 fold change	p-adj	P-value DFS
THBS1	1.51	2.83E-08	0.001
FAM83D	1.48	4.47E-03	0.363
CXCR4	1.27	1.07E-22	0.987
E2F8	1.27	2.87E-03	0.385
EREG	1.14	7.37E-09	0.011
IL6	1.04	1.64E-18	0.054
HEY1	1.02	9.43E-05	0.116
DEPDC7	0.96	4.24E-02	0.994
CIITA	0.91	1.63E-03	0.002
JAG1	0.87	1.03E-13	0.001
NXPH3	0.87	6.95E-03	0.764
TTK	0.85	3.47E-02	0.540
HTR2B	0.83	3.97E-02	0.368
CBR3	0.82	2.10E-02	0.553
HJURP	0.82	1.81E-03	0.410
AATK	0.81	5.93E-03	0.772
RUNX2	0.78	4.47E-03	0.621
IL12B	0.77	4.30E-02	0.608
CHST7	0.77	1.56E-05	0.055
ZFP36L1	0.76	4.73E-12	0.178
TCF7L2	0.74	3.57E-06	0.740
ABI3	0.72	2.41E-04	0.055
RAB33A	0.71	3.04E-02	0.624
GPR68	0.70	5.09E-04	0.867
FZD1	0.69	2.72E-05	0.304
MAP3K14	0.67	4.81E-03	0.472
ADA	0.66	4.04E-05	0.442
PID1	0.65	1.60E-05	0.022
PTGER2	0.65	1.14E-02	0.112
CMKLR1	0.64	1.10E-02	0.500
TAOK3	0.64	7.07E-06	0.574
RIN3	0.64	4.05E-06	0.079
MAEA	0.63	1.09E-06	0.070
SLC16A6	0.63	7.52E-05	0.530
CDCA8	0.62	3.74E-02	0.665
SMAD3	0.62	2.92E-02	0.446
CCR7	0.61	5.20E-06	0.071
MEF2C	0.60	1.89E-03	0.390
SERPINB9	0.60	2.55E-03	0.786
SYNJ2	0.59	2.52E-02	0.900
CDKN2AIP	0.59	1.65E-05	0.798
GPR157	0.59	1.32E-02	5.6^-5
CSF2	0.55	8.31E-04	0.605
UACA	0.53	2.55E-03	0.256
NFKBID	0.53	3.43E-07	0.812
SIAH2	0.51	5.20E-07	0.445
BTG1	0.51	2.51E-03	0.138
CXXC5	0.50	8.03E-05	0.282

C

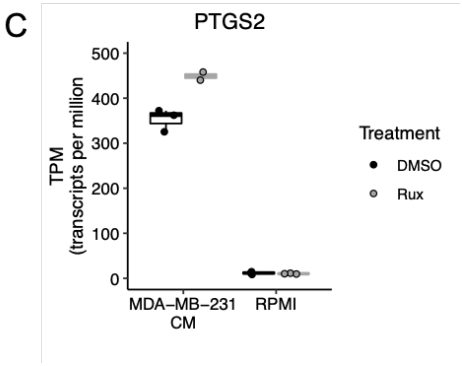


Fig. S6. JAK inhibition in macrophages leads to upregulation of pro- and anti-tumor genes. A. Analysis of M1/M2 target genes in PBMC-derived macrophages treated with conditioned medium from MDA-MB-231 cells compared with PBMC-derived macrophages treated with RPMI control medium. Error bars indicate SEM. B. List of genes that were significantly upregulated in PBMC-derived macrophages treated with tumor cell conditioned medium and ruxolitinib compared with tumor cell conditioned medium and DMSO. P-value for disease-free survival was calculated for basal-like breast cancers using an online database (breastmark). C. Raw values of PTGS2 in the indicated samples from the RNA-seq analysis.

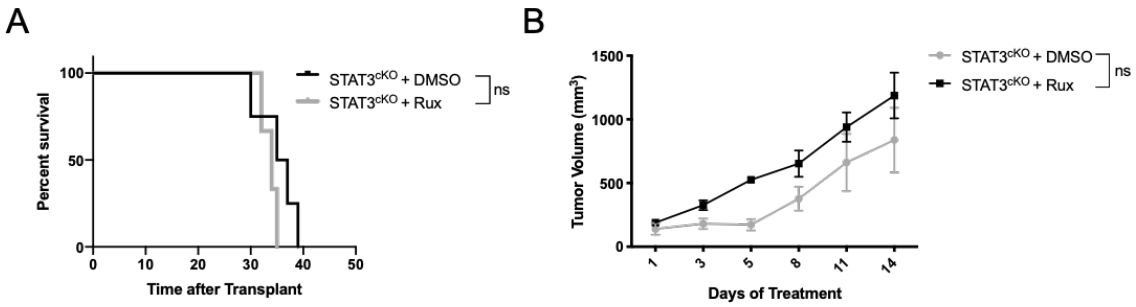


Fig. S7. Ruxolitinib does not impact tumor growth or survival in STAT3^{cKO} mice. A. Kaplan-Meier survival curve and (B) tumor volume growth curve of 4T1 cells transplanted into STAT3^{cKO} mice given DMSO as vehicle control (n=4) or Ruxolitinib (n=3).