SUPPLEMENTARY MATERIALS

Identification of the JNK-Active Triple-Negative Breast Cancer Cluster Associated with an Immunosuppressive Tumor Microenvironment

Authors: Takashi Semba[1,2], Xiaoping Wang[1,2], Xuemei Xie[1,2], Evan N. Cohen[2,3], James M. Reuben[2,3], Kevin N. Dalby[4,5], James P. Long[6], Lan Thi Hanh Phi[1,2], Debu Tripathy[1], and Naoto T Ueno[1,2]*

[1]Section of Translational Breast Cancer Research, Department of Breast Medical Oncology, The University of Texas MD Anderson Cancer Center
[2]Morgan Welch Inflammatory Breast Cancer Research Program and Clinic, The University of Texas MD Anderson Cancer Center
[3]Department of Hematopathology, The University of Texas MD Anderson Cancer Center
[4]Division of Chemical Biology and Medicinal Chemistry, College of Pharmacy, The University of Texas at Austin
[5]Department of Oncology, Dell Medical School, The University of Texas at Austin
[6]Department of Biostatistics, Division of Basic Science Research, The University of Texas MD Anderson Cancer Center

*Correspondence:
Naoto T. Ueno, M.D., Ph.D., F.A.C.P.
Professor of Medicine
Nylene Eckles Distinguished Professor in Breast Cancer Research
Chief, Section of Translational Breast Cancer Research
Executive Director, Morgan Welch Inflammatory Breast Cancer Research Program
Email: nueno@mdanderson.org, D: 713-792-8754, C: 713-401-9238
Department of Breast Medical Oncology
1515 Holcombe Boulevard, Unit 1354, Houston, Texas 77030

Supplementary Methods

Mouse experiments

For JNK-IN-8 treatment experiments, 5×10^5 E0771 cells were injected into mfps of C57BL/6 mice, and 1×10^4 4T1.2 cells were injected into mfps of BALB/c mice. When the tumors were palpable, mice were randomized for vehicle or JNK-IN-8 (Selleck Chemicals, 30 mg/kg)

treatment daily intraperitoneal (ip) injections. For CCL2 rescue experiments, 1×10^4 4T1.2 cells were injected into mfps of BALB/c mice. After 7 days from the inoculation of tumor cells, mice were randomized for treatment with vehicle, JNK-IN-8 (30 mg/kg, ip daily) + PBS, or JNK-IN-8 + rmCCL2. PBS or rmCCL2 was administered by an osmotic pump implanted subcutaneously in the backs of mice at the rate of 10 ng/h as previously described [36]. To evaluate the impact of CCL2 secreted from TAMs, 5×10^5 E0771 cells were injected into mfps of *Ccl2-RFP*$^{flox/flox}$ mice and *LyzM-Cre*:*Ccl2-RFP*$^{flox/flox}$ mice.

The tumor volumes were calculated every 2 or 3 days by the following formula: volume = short axis^2 x long axis/2 (mm^3). Mice were anesthetized, and blood was collected before and 23 days after tumor cell inoculation. Blood from mice was withdrawn from the orbital sinus and collected into tubes followed by centrifugation for 5 min at 10,000 *g* at room temperature (RT); then, the serum was collected into new tubes and stored at –80°C. At the end of the experiment, tumors and lungs of mice were collected from each tumor-bearing mouse and subjected to further analysis. For flow cytometry analysis, single cells from tumors were prepared. Tumors were minced into small pieces and digested with Accumax (STEMCELL Technologies) for 60 min at 37°C with constant shaking. The cells were sequentially filtered through 100-□m and 40□m cell strainers (Falcon) and centrifuged at 2500 rpm for 10 min. Cell pellets were then resuspended with red blood cell lysis buffer (Sigma) and incubated for 1 min at 37°C. The cells were centrifuged at 2500 rpm for 5 min at 4°C, and the pellets were subjected to further analysis. For histological analysis, tumors and lungs from mice were fixed in 10% neutral buffered formalin (StatLab Medical Products) and embedded in paraffin. Pieces of each tumor were lysed in lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate acid, 0.1% sodium dodecyl sulfate [SDS]) for protein extraction or lysis buffer provided in the

PureLink RNA Mini Kit (Invitrogen) for RNA extraction.

Patient samples

A microarray of tumor tissues from TNBC patients was purchased from US Biomax. Serum levels of CCL2 from TNBC patients and healthy donors who had participated in previous diagnostic protocols at MD Anderson were analyzed using the Millipore Milliplex Human Cytokine/Chemokine Panel I, a multiplex assay kit (Millipore), and a Luminex Analyzer 100. This study was approved by the MD Anderson Institutional Review Board (IRB) as LAB090347, LAB09-049, 2005-0243, ID02-052, 2006-1072, ID02-458, LAB03-0479, LAB05-0083, ID99-231, LAB08-0231, and LAB08-0199.

Cell culture

4T1.2 cells were obtained from Robin L. Anderson (School of Cancer Medicine, La Trobe University, Australia). E0771 cells were purchased from CH3 BioSystems. E0771 cells were injected into mfps of C57BL/6 mice to obtain highly metastatic populations, and cells isolated from metastatic lung foci were used in this study. THP-1 cells were purchased from American Type Culture Collection. Ctrl, JNK1-KO, and JNK2-KO THP-1 cells were obtained from Geoffrey Bartholomeusz (MD Anderson Cancer Center). 4T1.2, E0771, and THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Sigma) in 5% CO_2 at 37°C. Buffy coats from healthy donors were purchased from Gulf Coast Regional Blood Center to obtain PBMCs. PBMCs were isolated from the buffy coats by Ficoll density gradient centrifugation. To obtain human primary macrophages, PBMCs were seeded in cell culture plates at a cell density of 5×10^5 cells per 1 cm^2 with RPMI-1640 medium supplemented with 10% FBS and 1%

antibiotic/antimycotic. Medium was aspirated after 4 hours to remove floating cells and changed with RPMI-1640 medium supplemented with 5% FBS and 10 ng/ml of human macrophage colony-stimulating factor (M-CSF; PeproTech). Cells were cultured for 6 days to differentiate into macrophages and used for further experiments. Bone marrow macrophages from *Ccl2RFP*$^{flox/flox}$ mice and *LyzM-Cre*:*Ccl2-RFP*$^{flox/flox}$ mice were obtained by culturing bone marrow cells from the femurs of each genotype on cell culture plates with RPMI-1640 medium supplemented with 10% FBS and 10 ng/ml of murine M-CSF (PeproTech) for 6 days. TNBC cell lines used in experiments to measure the concentration of CCL2 in CM (Supplementary Figure 5B) were as follows: BT-20 (ATCC), HCC70 (ATCC), BT-549 (ATCC), Hs578T (ATCC), MDA-MB-468 (ATCC), MDA-MB-231 (ATCC), HCC1937 (ATCC), CAL51 (DSMZ-German Collection of Microorganisms), and SUM159 (Asterand Bioscience). HCC70, BT-549, and HCC1937 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% antibiotic/antimycotic. CAL51 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% antibiotic/antimycotic. SUM159 cells were cultured in Nutrient Mixture F12 Ham (F12) (Sigma) supplemented with 5% FBS, 4 mg/ml insulin (Gibco), 2 mg/ml hydrocortisone (Sigma), and 1% antibiotic/antimycotic. BT-20, Hs578T, MDA-MB-468, and MDA-MB-231 cells were cultured in DMEM/F12 (Sigma) supplemented with 10% FBS and 1% antibiotic/antimycotic.

Flow cytometry

Single-cell suspensions from tumor samples or cultured cells were blocked with TruStain FcX (BioLegend) for murine cells or anti-human IgG Fc antibody (HP6017, BioLegend) for human primary macrophages in FACS buffer (2% FBS, 2 mM EDTA PBS) to minimize the nonspecific expression of surface proteins. Cells were subsequently stained with LIVE/DEAD Aqua fixable

dead cell staining dye (Life Technologies) for 30 min on ice. For analyzing TNBC mouse tumor samples, cells were stained in FACS buffer for 30 min on ice with the antibodies targeting cell surface antigens; CD45-FITC (30-F11, BioLegend), CD3-PerCP/Cy5.5 (17A2, BioLegend), CD4-Pacific Blue (RM4-5, BioLegend), CD8-PE (53-6.7, BioLegend), CD25-APC/Fire750 (PC61, BioLegend), and F4/80-Alexa700 (BM8, BioLegend). After 2 washes with FACS buffer, cells were fixed and permeabilized using Foxp3/Transcription Factor Staining Buffer (eBioscience) for 30 min on ice in the dark. Cells were then washed twice with FACS buffer and stained with FOXP3-PE/Cy7 (FJK-16s, eBioscience) and CCL2-APC (2H5, BioLegend). For analyzing tumors or bone marrow-derived macrophages from *Ccl2-RFP*$^{flox/flox}$ mice and *LyzM-Cre*:*Ccl2-RFP*$^{flox/flox}$ mice, cells were stained in FACS buffer for 30 min on ice with CD45-Pacific Blue (30-F11, BioLegend), CD3-APC/Fire750 (17A2, BioLegend), CD19-APC (1D3/CD19, BioLegend), and F4/80-Alexa700. For analyzing human primary macrophages, cells were stained with human CD68-PerCP/Cy5.5 (Y1/82A, BioLegend) in FACS buffer for 30 min on ice. Detailed information about the antibodies is provided in table S2. Samples were analyzed using a Gallios flow cytometer (Beckman Coulter), and appropriate single-color controls were used for compensation. Data were analyzed by FlowJo software.

Tissue histology

Paraffin-embedded samples were cut with a cryotome and placed on slides for hematoxylin-eosin (H&E) or multiplex IHC staining. To measure the extent of lung metastasis, sections of each lung from tumor-bearing mice were subjected to H&E staining. Whole images of lungs were scanned using a PathScan Enabler IV scanner (Meyer), and the tumor area or total lung area was quantified by ImageJ software.

For multiplex IHC staining, we used the Opal 7-Color IHC kit (AKOYA) to generate multiple immunofluorescent staining slides according to the manufacturer's instructions. Slides were baked at 65°C for 1 hour, dewaxed with xylene, and rehydrated through a graded series of ethanol solutions. After rehydration, slides were fixed in 10% neutral buffered formalin for 20 min. Antigen retrieval was done by microwave treatment with AR6 buffer (AKOYA). After slides were cooled down at RT, slides were rinsed with Tris-buffered saline with Tween 20 (TBST) (Santa Cruz) and incubated with Antibody Diluent/Block buffer (AKOYA) for 10 min at RT. Slides were then incubated with one of the following primary antibodies for 1 hour at RT or overnight at 4°C: F4/80 (CI:A3-1, Bio-Rad), FOXP3 (FJK-16s, eBioscience), and RFP (Abcam) for mouse tumor tissue staining; CD8 (Abcam), CD68 (SP251, Sigma), FOXP3 (236A/E7, Abcam), CCL2 (2D8, LS Bio), pC-JUN (Ser73) (D47G9, Cell Signaling), and CK7 (OV-TL 12/30, Dako) for TNBC tumor tissue microarray staining. Detailed information about antibodies is provided in table S3. After washing three times with TBST, slides were incubated with Opal Polymer HRP Ms + Rb (AKOYA) for 10 min at RT. After washing three more times with TBST, the slides were then incubated at RT for 10 min with one of the following reagents provided in the kit: Opal 480, Opal 520, Opal 570, Opal 620, Opal 690, and Opal TSA-DIG. To strip the primary-secondary-HRP complex, slides were boiled with AR6 buffer using a microwave followed by cooling and TBST washes. The cycle from blocking to antibody-complex stripping was repeated until all targets of interest above were developed. Opal TSA-DIG staining was performed at the last cycle, and the slides were then incubated with Opal 780 at RT for 10 min after microwave treatment. After three washes with TBST, the slides were counterstained with DAPI for 5 min at RT and mounted with ProLong Diamond Antifade Mountant (Thermo Fisher).

We used the Vectra Polaris imaging system (PerkinElmer) to scan the slides and analyzed the images using Phenochart and InForm software (Perkin Elmer). Five random areas on each sample were analyzed for mouse experiments. For the TNBC tumor tissue microarray, the entire spot of each patient sample was analyzed.

ELISA

Mouse CCL2, CXCL1, and human CCL2 protein levels were quantified using DuoSet ELISA kits (R&D Systems). Data were collected using a VICTOR X plate reader (PerkinElmer). All experiments were performed according to the manufacturer's instructions.

Cell viability assay

E0771 or 4T1.2 cells were plated in 96-well plates. The next day, cells were treated with various concentrations of JNK-IN-8 (Selleck Chemicals). After 96 hours, cell viability was determined using CellTiter-Blue (Promega) and a VICTOR X plate reader (PerkinElmer) according to the manufacturer's instructions.

Cytokine/chemokine array

A Mouse Chemokine Array C1 kit (RayBiotech) was used for screening chemokine levels in tumors from vehicle- or JNK-IN-8-treated mice. We used 200 □g of protein from each tumor sample to detect chemokines according to the manufacturer's instructions.

Detection of cytokines and chemokines produced from DMSO- or JNK-IN-8 treated M2 THP-1 cells was performed using the Milliplex Human Cytokine/Chemokine Panel I (Millipore), a multiplex assay kit, and a Luminex Analyzer 100. Obtained data were standardized by converting them into z-scores using Spotfire software (TIBCO). In addition, hierarchical

clustering and heatmap creation were performed using ClustVis (https://biit.cs.ut.ee/clustvis/) to display the chemokine levels.

JNK-IN-8 treatment for THP-1 cells and human primary macrophages and CCL2 assays

THP-1 cells (4×10^6) were plated in a 10-cm cell culture dish with RPMI-1640 medium supplemented with 10% FBS and 50 ng/ml PMA (Sigma) for 16 hours to differentiate into M0 THP-1 cells. To generate M2 THP-1 cells, M0 THP-1 cells were incubated with RPMI1640 medium supplemented with 10% FBS, 20 ng/ml IL-4 (PeproTech), and 20 ng/ml IL-13 (PeproTech) for 48 hours. After differentiation, M0 or M2 THP-1 cells were washed with PBS three times and incubated with DMSO or JNK-IN-8 (2.5 or 5 μM) containing 10% FBS RPMI1640 for 48 hours. CCL2 or other cytokines/chemokines in CM from each group were analyzed by ELISA or Luminex assay.

Ctrl, JNK1-KO, or JNK2-KO THP-1 cells (4×10^6) were plated in a 10-cm cell culture dish with RPMI-1640 medium supplemented with 10% FBS and 50 ng/ml PMA for 16 hours. After three PBS washes, cells were incubated with 10% FBS RPMI1640 for 48 hours, and the CCL2 protein level in CM from each cell line was analyzed by ELISA.

Human primary macrophages were washed with PBS three times and incubated with DMSO or JNK-IN-8 (1 or 2.5 μM) containing 10% FBS RPMI-1640 for 48 hours, and CCL2 protein levels in the CM from each treatment group were analyzed by ELISA.

The results from ELISA analysis were normalized by the cell numbers at the endpoint of the experiments.

siRNA transfection in primary macrophages

To knock down the *JNK1, JNK2,* or *C-JUN* gene in human primary macrophages, we transfected siRNA targeting each gene into cells as previously described [1]. Briefly, PBMCs isolated from buffy coats were seeded and cultured at a cell density of 5×10^5 cells per 1 cm^2 with 5% FBS RPMI-1640 supplemented with 10 ng/ml of human M-CSF for 5 days to differentiate cells into macrophages. Cells were then transfected with non-targeting siRNA (Dharmacon) or siRNA targeting the *JNK1, JNK2,* or *C-JUN* gene (Sigma) at a final siRNA concentration of 200 nM using HiPerFect Transfection Reagent (Qiagen). After 48 hours, cells were washed with PBS three times and incubated with 5% FBS RPMI-1640 for 48 hours. CCL2 protein levels in CM from each group were analyzed by ELISA, and the results from ELISA analysis were normalized by the cell numbers at the endpoint of experiments. The silencing of each target gene was validated by Western blot analysis.

qPCR

For analyzing *Ifng*, *Il2,* and *Ccl2* mRNA expression levels of mouse tumor samples, RNAs from each mouse tumor sample were extracted and purified using a PureLink RNA Mini Kit. cDNAs were synthesized from 1 □g of total RNA using amfiRivert cDNA Synthesis Platinum Master Mix (genDEPOT). Quantitative PCR (qPCR) was performed using primers as follows: *Ifng* forward, 5'-ATGAACGCTACACACTGCATC-3'; *Ifng* reverse, 5'-CCATCCTTTTGCCAGTTCCTC-3'; *Il2* forward, 5'-TGAGCAGGATGGAGAATTACAGG-3'; *Il2* reverse, 5'-GTCCAAGTTCATCTTCTAGGCAC-3'; *Ccl2* forward, 5'-TTAAAAACCTGGATCGGAACCAA-3'; *Ccl2* reverse, 5'-

GCATTAGCTTCAGATTTACGGGT-3'; *Actb* forward, 5'-GGCTGTATTCCCCTCCATCG-3'; *Actb* reverse, 5'-CCAGTTGGTAACAATGCCATGT-3'. mRNA expression levels of each gene were normalized by that of *Actb*.

For analyzing DNA obtained from the ChIP assay, purified DNA was used as a template, and qPCR was performed using the following primers: *CCL2* forward, 5'-ACCCTTCACCTTCCCTGTGTTTACT-3'; *CCL2* reverse, 5'CATAGGCTTCTGAGTGTTGGAAGCA-3'; negative ctrl forward, 5'-CTCCCAAATTGCTGGGATTA-3'; negative ctrl reverse, 5'-ATTCCAGGCACCACAAAAAG-3'. In addition, the C-JUN binding site in the promoter region of the *CCL2* gene was analyzed using Genome Browser (https://genome.ucsc.edu/index.html) (University of California, Santa Cruz) and JASPAR (http://jaspar.genereg.net/).

iTaq Universal SYBR Green Supermix (Bio-Rad) and a CFX96 Real-Time PCR Detection System (Bio-Rad) were used for qPCR reaction and data collection according to the manufacturer's instructions.

Western blot analysis

Cells were lysed in lysis buffer supplemented with protease inhibitor and phosphatase inhibitor cocktail (Bimake.com). We mixed 30 ug of protein from each sample with NuPAGE LDS Sample Buffer (Thermo Fisher Scientific) and boiled it at 95°C for 5 min. Proteins were fractioned by SDS-PAGE on 4% to 12% NuPAGE Bis-Tris gels (Thermo Fisher Scientific) and transferred to Immun-Blot PVDF membrane (Bio-Rad). Proteins of interest were probed using anti-JNK (Cell Signaling), anti-phospho-JNK (G9, Cell signaling), anti-JNK1 (2C6, Cell Signaling), anti-JNK2 (Cell Signaling), anti-C-JUN (60A8, Cell Signaling), or anti-β-actin antibody (AC-15, Sigma). Horseradish peroxidase (HRP)-conjugated anti-rabbit (Invitrogen) or

anti-mouse antibody (Invitrogen) were used as secondary antibodies. HRP on immunoblots were detected using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) or SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) and iBright FL1000 Imaging System (Thermo Fisher Scientific).

Chromatin immunoprecipitation assay

Human primary macrophages (1×10^7) were fixed with 16% formaldehyde (Thermo Fisher Scientific) for 10 min at RT. We added 2.5 M glycine to a final concentration of 125 mM and incubated it for 5 min at RT. After two washes with cold PBS, cells were collected and snapfrozen in liquid nitrogen. DNA fragments were obtained using a chromatin extraction kit (Abcam) according to the manufacturer's instructions, followed by a 5-fold dilution with Pierce IP Lysis Buffer (Thermo Fisher Scientific) and incubation with rabbit IgG (Cell Signaling) or anti-C-JUN antibody (60A8, Cell Signaling) overnight at 4°C. Pre-washed nProtein A/Protein G Sepharose 4 Fast Flow beads mix (GE Healthcare) were added in samples and incubated for 3 hours at 4°C. After centrifugation and removal of supernatant, samples were then washed with low-salt buffer (0.1% SDS, 1% TritonX-100, 2 mM EDTA, 20 mM Tris-HCL [pH 8.0], 150 mM NaCl), high-salt buffer (0.1% SDS, 1% TritonX-100, 2 mM EDTA, 20 mM Tris-HCL [pH 8.0], 500 mM NaCl), and LiCl Immune Complex Wash Buffer (Millipore Sigma). DNA was eluted with elution buffer (1% SDS, 100 mM $NaHCO_3$) followed by RNase (Thermo Fisher Scientific) and proteinase K (ApexBio) treatment and purified using a PureLink PCR purification kit (Invitrogen).

Tumor-infiltrating lymphocyte analysis

Digital pathology images for 93 TNBC patients in the TCGA database were obtained from the

Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov/) in January 2021. A Vectra Polaris imaging system was used to scan an H&E-stained tissue microarray slide purchased from US Biomax. The analysis of tumor-infiltrating lymphocytes (TILs) was performed using QuPath 0.2.3 [2] as previously described [3]. We annotated detected cells as tumor cells, TILs, and stromal cells and optimized an algorithm to classify cells based on the annotation by a machinelearning method. Detected counts of TILs and tumor cells were used to calculate eTILs% as follows: (TILs count/[TILs count + tumor cells count]) x 100.

Bioinformatical analysis

RPPA data of 95 TNBC patients in the TCGA database were obtained from The Cancer Proteome Atlas (https://tcpaportal.org/tcpa/). RNA-sequencing (RNA-seq) data of the corresponding 94 patients in the RPPA dataset were obtained from the Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov/) in January 2019; RNA-seq data for one patient were not available. mRNA expression data for 299 TNBC patients in the METABRIC database were obtained from cBioportal (https://www.cbioportal.org/). For survival analysis in TNBC patients based on protein expression, we used the tool Kaplan-Meier Plotter [4] (https://kmplot.com/analysis/).

JNK^{high} and JNK^{low} subpopulations of TNBC patients were classified based on the pJNK and pC-JUN expression levels, with the mean values used for the cutoff points. RNA-seq data of each subpopulation were then analyzed using the iDEP web application [5] (http://bioinformatics.sdstate.edu/idep/).

Differentially expressed genes between the JNK^{high} and JNK^{low} subpopulations were selected with a controlled false positive rate of ≤ 0.1, and up- and downregulated genes were

selected at a minimum 2-fold change. Pathways enriched in selected differentially expressed genes were examined by GO analysis.

To predict immune cell infiltration in the TME, we analyzed RNA-seq data of JNK^{high} and JNK^{low} TNBC patients using the CIBERSORT tool (https://cibersort.stanford.edu/). The result was displayed as a heatmap created by using Spotfire.

References

1. Troegeler A, Lastrucci C, Duval C, *et al*. An efficient siRNA-mediated gene silencing in primary human monocytes, dendritic cells, and macrophages. Immunol Cell Biol. 2014;92(8):699-708.

2. Bankhead P, Loughrey MB, Fernandez JA, *et al*. QuPath: Open source software for digital pathology image analysis. Sci Rep. 2017;7(1):16878.

3. Acs B, Ahmed FS, Gupta S, et al. An open source automated tumor infiltrating lymphocyte algorithm for prognosis in melanoma. Nat Commun. 2019;10(1):5440.

4. Nagy A, Lanczky A, Menyhart O, *et al*. Validation of miRNA prognostic power in hepatocellular carcinoma using expression data of independent datasets. Sci Rep. 2018;8(1):9227.

5. Ge SX, Son EW, and Yao R. iDEP: an integrated web application for differential expression and pathway analysis of RNA-Seq data. BMC Bioinformatics. 2018;19(1):534.

Supplementary Tables

Supplementary Table 1. Differentially up-regulated genes in the JNK^{high} subgroup of TNBC patients (TCGA dataset).

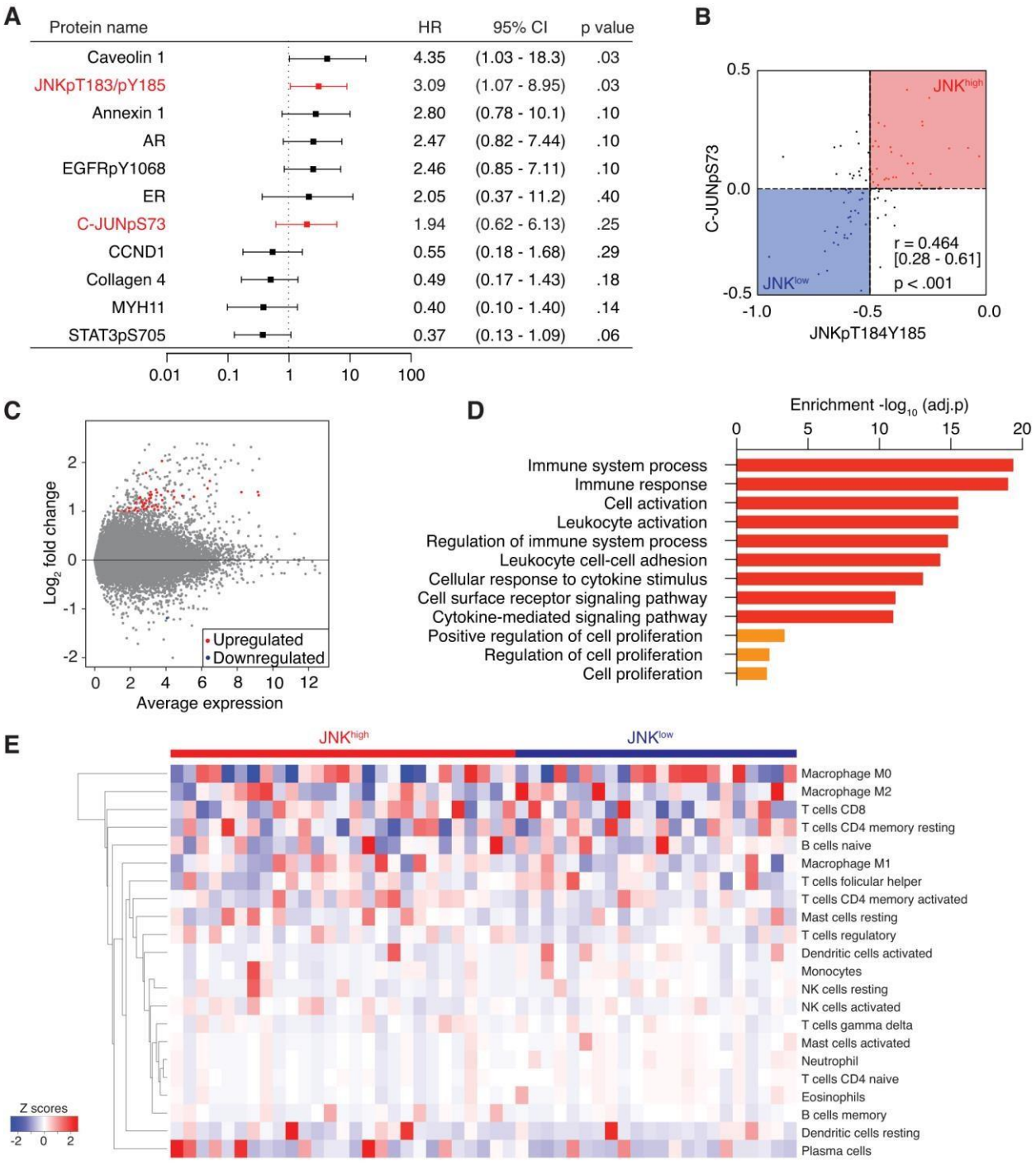
Ensembl gene ID	Gene name	Gene symbol
ENSG00000204472	allograft inflammatory factor 1	*AIF1*

ENSG00000128284	apolipoprotein L3	*APOL3*
ENSG00000156127	basic leucine zipper ATF-like transcription factor	*BATF*
ENSG00000130303	bone marrow stromal cell antigen 2	*BST2*
ENSG00000172724	C-C motif chemokine ligand 19	*CCL19*
ENSG00000158481	CD1c molecule	*CD1C*
ENSG00000139193	CD27 molecule	*CD27*
ENSG00000104894	CD37 molecule	*CD37*
ENSG00000167286	CD3d molecule	*CD3D*
ENSG00000198851	CD3e molecule	*CD3E*
ENSG00000117091	CD48 molecule	*CD48*
ENSG00000169442	CD52 molecule	*CD52*
ENSG00000143119	CD53 molecule	*CD53*
ENSG00000019582	CD74 molecule	*CD74*
ENSG00000102879	coronin 1A	*CORO1A*
ENSG00000077984	cystatin F	*CST7*
ENSG00000186810	C-X-C motif chemokine receptor 3	*CXCR3*
ENSG00000147443	docking protein 2	*DOK2*
ENSG00000105246	Epstein-Barr virus induced 3	*EBI3*
ENSG00000185862	ecotropic viral integration site 2B	*EVI2B*
ENSG00000149781	fermitin family member 3	*FERMT3*
ENSG00000130755	glia maturation factor gamma	*GMFG*
ENSG00000145649	granzyme A	*GZMA*
ENSG00000204257	major histocompatibility complex, class II, DM alpha	*HLA-DMA*
ENSG00000226264	major histocompatibility complex, class II, DM beta	*HLA-DMB*
ENSG00000204252	major histocompatibility complex, class II, DO alpha	*HLA-DOA*
ENSG00000168384	major histocompatibility complex, class II, DP alpha 1	*HLA-DPA1*
ENSG00000215048	major histocompatibility complex, class II, DP beta 1	*HLA-DPB1*
ENSG00000196735	major histocompatibility complex, class II, DQ alpha 1	*HLA-DQA1*
ENSG00000196610	major histocompatibility complex, class II, DQ beta 2	*HLA-DQB2*
ENSG00000204287	major histocompatibility complex, class II, DR alpha	*HLA-DRA*
ENSG00000196126	major histocompatibility complex, class II, DR beta 1	*HLA-DRB1*
ENSG00000110324	interleukin 10 receptor subunit alpha	*IL10RA*
ENSG00000147168	interleukin 2 receptor subunit gamma	*IL2RG*
ENSG00000140968	interferon regulatory factor 8	*IRF8*
ENSG00000005844	integrin subunit alpha L	*ITGAL*
ENSG00000204487	lymphotoxin beta	*LTB*
ENSG00000131401	napsin B aspartic peptidase, pseudogene	*NAPSB*
ENSG00000100365	neutrophil cytosolic factor 4	*NCF4*
ENSG00000105374	natural killer cell granule protein 7	*NKG7*
ENSG00000177989	outer dense fiber of sperm tails 3B	*ODF3B*
ENSG00000179715	PC-esterase domain containing 1B	*PCED1B*
ENSG00000128340	Rac family small GTPase 2	*RAC2*
ENSG00000122122	SAM and SH3 domain containing 3	*SASH3*
ENSG00000110876	selectin P ligand	*SELPLG*
ENSG00000066336	Spi-1 proto-oncogene	*SPI1*
ENSG00000167895	transmembrane channel like 8	*TMC8*
ENSG00000211772	T cell receptor beta constant 2	*TRBC2*
ENSG00000211753	T cell receptor beta variable 28	*TRBV28*
ENSG00000015285	WASP actin nucleation promoting factor	*WAS*

Supplementary Table 2. Antibodies used in this study.

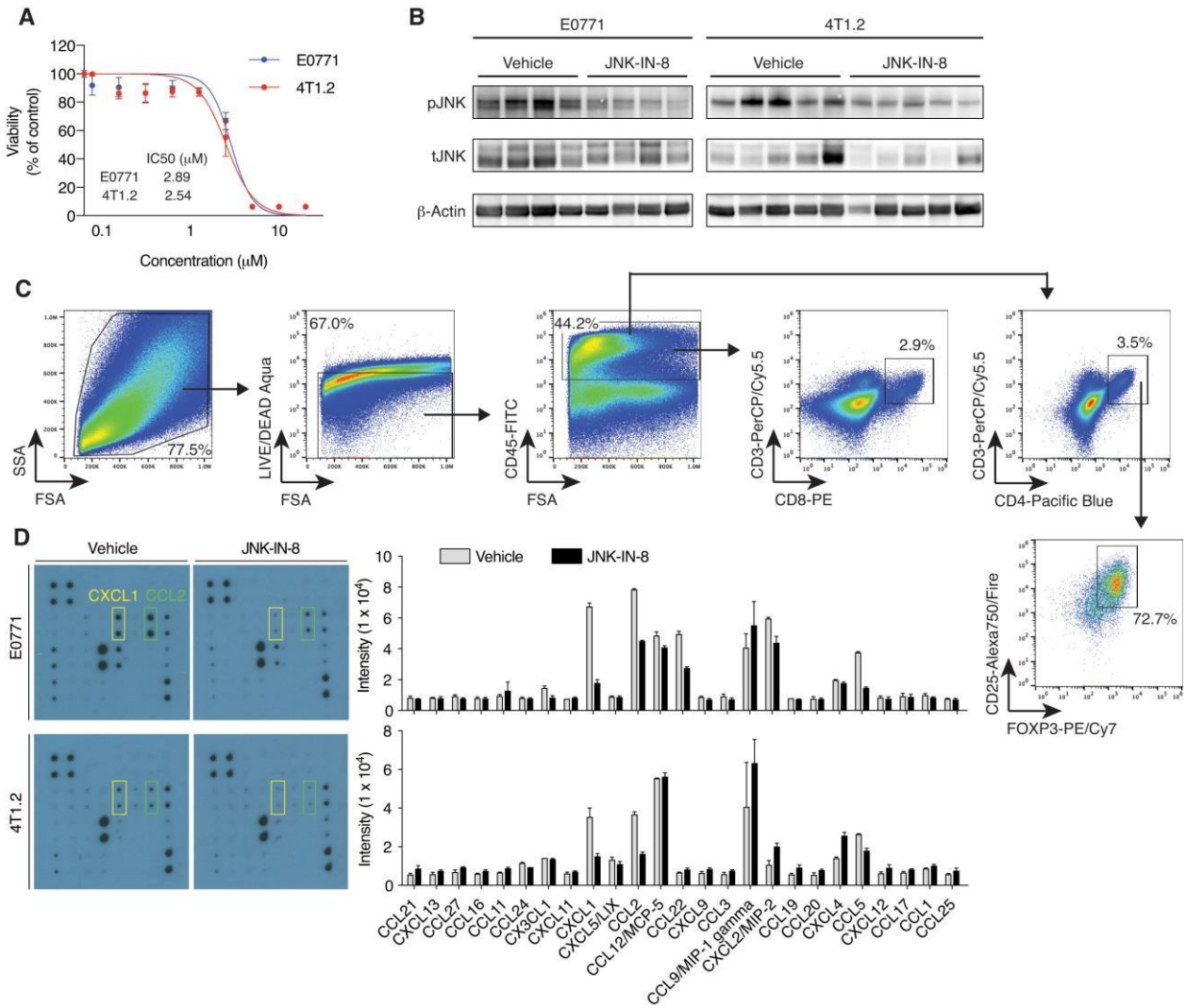
Antibody	Clone	Source	Catalog number
TruStain FcX	93	BioLegend	101320
anti-human IgG Fc antibody	HP6017	BioLegend	409302
anti-mouse CD45-FITC antibody	30-F11	BioLegend	103108
anti-mouse CD45-PE antibody	30-F11	BioLegend	103106
anti-mouse CD45-PerCP/Cy5.5 antibody	30-F11	BioLegend	103131
anti-mouse CD45-PE/Cy7 antibody	30-F11	BioLegend	103114
anti-mouse CD45-APC antibody	30-F11	BioLegend	103112
anti-mouse CD45-Alexa700 antibody	30-F11	BioLegend	103128
anti-mouse CD45-APC/750Fire antibody	30-F11	BioLegend	103153
anti-mouse CD45-Pacific Blue antibody	30-F11	BioLegend	103126
anti-mouse CD3-PerCP/Cy5.5 antibody	17A2	BioLegend	100218
anti-mouse CD3-APC/750Fire antibody	17A2	BioLegend	100247
anti-mouse CD4-Pacific Blue antibody	RM4-5	BioLegend	100531
anti-mouse CD8-PE antibody	53 -6.7	BioLegend	100707
anti-human CD8 polyclonal antibody		Abcam	ab4055
anti-mouse CD19-APC antibody	1D3/CD19	BioLegend	152409
anti-mouse CD25-APC/750Fire antibody	PC61	BioLegend	102054
anti-human CD68-PerCP/Cy5.5 antibody	Y182A	BioLegend	333810
anti-human CD68 antibody	SP251	Sigma	SAB5500070
anti-mouse F4/80-Alexa700 antibody	BM8	BioLegend	123130
anti-mouse F4/80 antibody	Cl:A3-1	Bio-Rad	MCA497
anti-mouse FOXP3-PE/Cy7 antibody	FJK-16s	eBioscience	25-5773-80
anti-human FOXP3 antibody	236A/E7	Abcam	ab20034
anti-mouse CCL2-APC antibody	2H5	BioLegend	505910
anti-human CCL2 antibody	2D8	LS Bio	C169178
anti-human CK7 antibody	OV -TL 12/30	Dako	M7018
anti-JNK polyclonal antibody		Cell Signaling	9252
anti-phospho-JNK antibody	G9	Cell Signaling	9255
anti-JNK1 antibody	2 C6	Cell Signaling	3708
anti-JNK2 polyclonal antibody		Cell Signaling	4672
anti-C-JUN antibody	60A8	Cell Signaling	9165
anti-phospho-C-JUN (Ser73) antibody	D47G9	Cell Signaling	3270
anti-b-Actin	AC -15	Sigma	5441
anti-Rabbit HRP-conjugated polyclonal IgG		Invitrogen	31460
anti-Mouse HRP-conjugated polyclonal IgG		Invitrogen	31430

Supplementary Figures



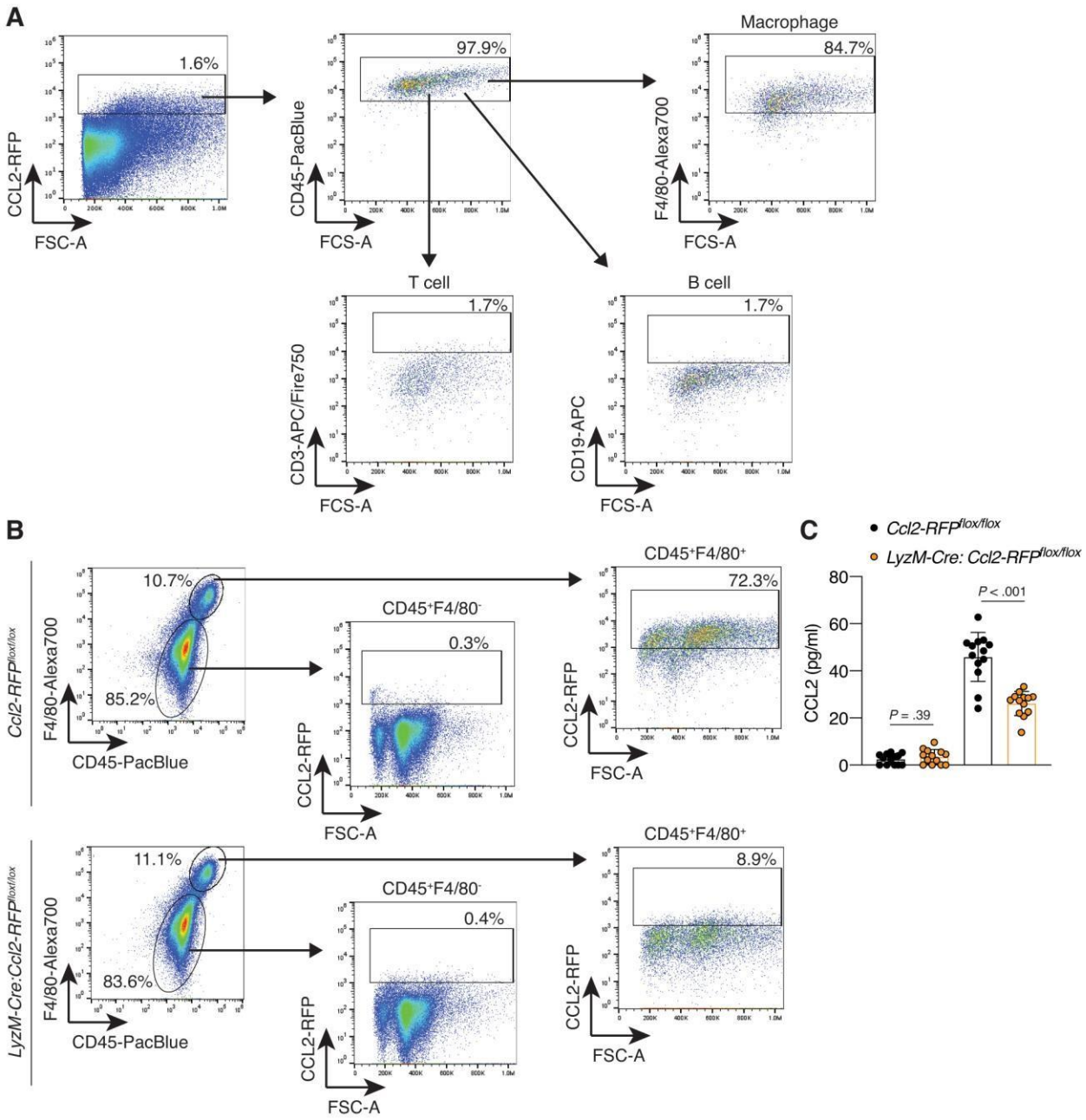
Supplementary Figure 1. The correlation between pJNK and an immunosuppressive TME in TNBC. (**A**) Hazard ratios (HRs) and 95% confidence intervals (CIs) for overall survival of TNBC patients from the TCGA cohort (n = 95), according to the expression level of proteins

enriched in cluster 1. A univariate Cox proportional-hazards model was used to estimate HRs and the significance of the comparison between high and low protein levels for overall survival. (**B**) Scatterplot of expression of pJNK and pC-JUN in 94 TNBC cases and definition of 2 subgroups. Spearman's correlation coefficient r, 95% confidence interval, and p-value are shown in the graph. (**C**) A ratio-intensity plot of the $\log_2$ fold change of all genes. Red or blue points indicate differentially expressed genes between JNK^{high} (n = 27) and JNK^{low} (n =22) subgroups with a fold change greater than 2 or less than −2, respectively. (**D**) Gene Ontology (GO) analysis of differentially upregulated genes in the JNK^{high} subgroup. (**E**) CIBERSORT output of JNK^{high} and JNK^{low} subgroups of TNBC patients from the TCGA cohort.


A
Viability (% of control)
Concentration (μM)
E0771
4T1.2
IC50 (μM)
E0771 2.89
4T1.2 2.54
B
E0771
4T1.2
Vehicle
JNK-IN-8
pJNK
tJNK
β-Actin
C
SSA
FSA
77.5%
LIVE/DEAD Aqua
67.0%
CD45-FITC
44.2%
CD3-PerCP/Cy5.5
CD8-PE
2.9%
CD4-Pacific Blue
3.5%
D
Vehicle
JNK-IN-8
E0771
4T1.2
CXCL1
CCL2
Intensity (1 x 10⁴)
CCL21
CXCL13
CCL27
CCL16
CCL11
CCL24
CX3CL1
CXCL11
CXCL1
CXCL5/LIX
CCL2
CCL12/MCP-5
CCL22
CXCL9
CCL3
CCL9/MIP-1 gamma
CXCL2/MIP-2
CCL19
CCL20
CXCL4
CCL5
CXCL12
CCL17
CCL1
CCL25
CD25-Alexa750/Fire
FOXP3-PE/Cy7
72.7%
E
CCL24
CCL2
CCL5
CXCL1
CXCL5
CX3CL1
CCL21
CCL27
CCL12
CXCL9
CXCL2
CCL20
CCL25
CCL17
CXCL13
CCL16
CCL22
CCL3
CCL19
CXCL4
CXCL12
CCL1
Down in 4T1.2
Down in E0771
F
E0771
4T1.2
CXCL1 (pg/ml)
P = .55
P = .79
Vehicle
JNK-IN-8
G
CCL2 (pg/ml)
P = .92
P = .83
P = .97
P = .70
P = .003
P < .001
BALB/c baseline
3 weeks
Vehicle (n =13)
JNK-IN-8 + PBS (n = 13)
JNK-IN-8 + rmCCL2 (n =13)

Supplementary Figure 2. The effect of JNK inhibitor treatment on TNBC tumor growth, metastasis, and the immune TME and the contributions of CCL2 to JNK-mediated immunosuppressive TME formation and tumor progression in TNBC. (**A**) Dose-response curves for E0771 and 4T1.2 cells with JNK-IN-8 treatment at the indicated concentrations. (**B**) Immunoblotting of phosphorylated JNK, total JNK, and □-actin in the tumor lysate from vehicle- or JNK-IN-8-treated E0771 and 4T1.2 mouse models. (**C**) Gating strategy of flow cytometry analysis for Treg and $CD8^+$ T cell subsets in tumors. Tumors from E0771 or 4T1.2 mouse models were dissociated to obtain a single-cell suspension and stained with antibodies. Cells were first gated to exclude debris by FSA vs. SSA, followed by gating for the LIVE/DEAD $Aqua^-$ and $CD45^+$ population. Tregs were identified by gating for $CD3^+CD4^+$ cells, followed by gating for $CD25^+FOXP3^+$ cells. $CD8^+$ T cells were identified by gating for $CD3^+CD8^+$ cells. (**D**) Antibody-based chemokine array analysis for 25 chemokines in the tumor lysate from vehicle- or JNK-IN-8-treated E0771 and 4T1.2 mouse models. The spots for CXCL1 and CCL2 are indicated using yellow and green rectangles, respectively. Each chemokine was detected in duplicate. The intensity of each spot was analyzed using Protein Array Analyzer for ImageJ software. The expression level of each protein was normalized by comparison with reference spots. (**E**) Diagram indicating chemokines downregulated in tumors from JNK-IN-8-treated 4T1.2 mice (red) and E0771 mice (blue) compared with vehicle-treated mice. (**F**) Quantification of CXCL1 concentration in tumors from E0771 (n =5) and 4T1.2 models (n =5). (**G**) Quantification of mean CCL2 concentration in sera from the vehicle (n =13), JNK-IN-8 + PBS (n =13), and JNK-IN-8 + rmCCL2 groups (n = 13). The CCL2 levels in sera collected before tumor injection are indicated as BALB/c baseline. Error bars represent ± SD. A two-tailed

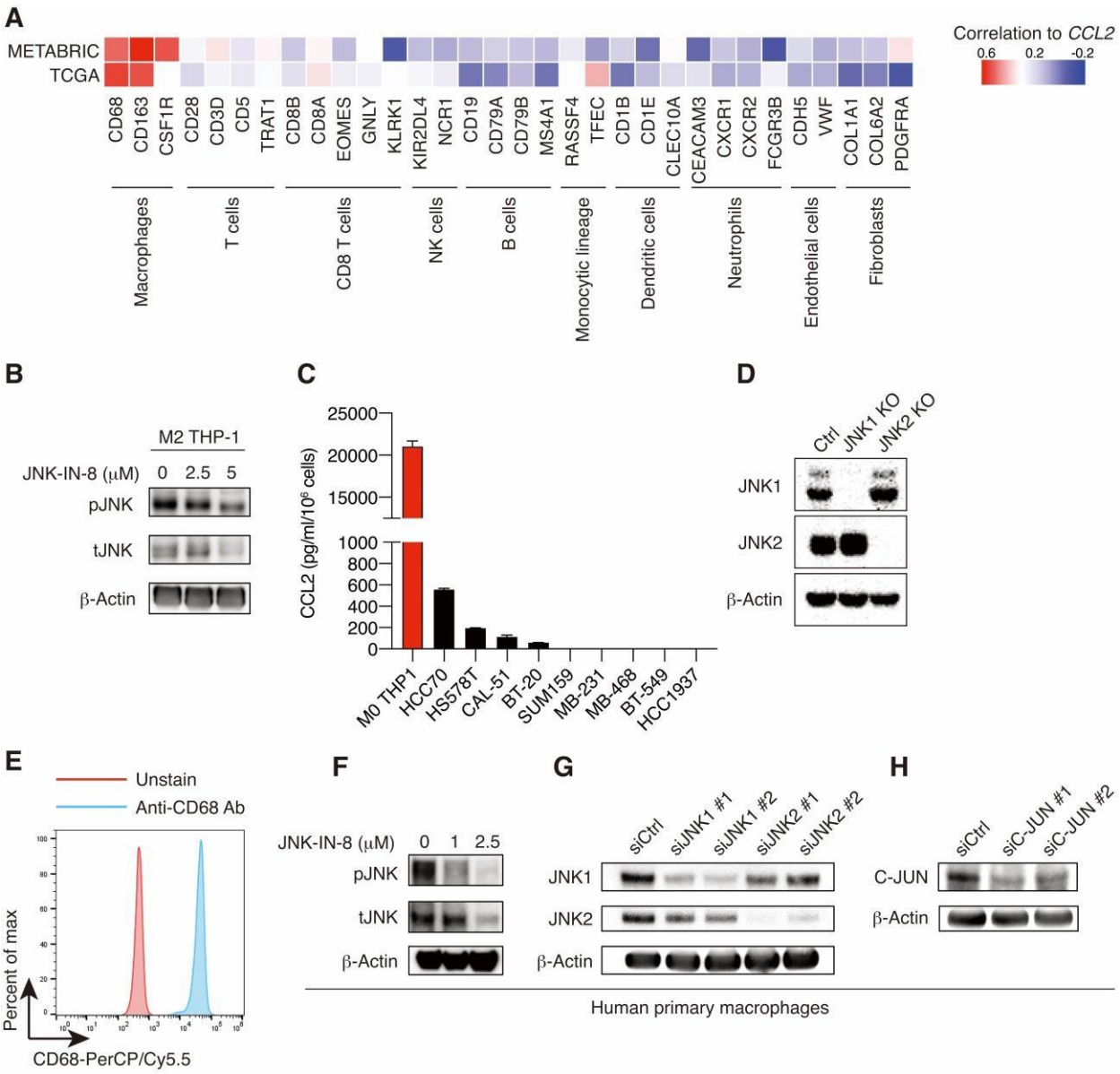
Student *t*-test in (**F**) and one-way ANOVA followed by Tukey multiple comparison test in (**G**) were used to calculate *P* values.



Supplementary Figure 3. The role of TAMs in producing CCL2 in the TME and TNBC tumor progression. (**A**) Gating strategy of flow cytometry analysis for Figure 4G. CCL2RFP^{+}CD45^{+} cells were analyzed for expression of F4/80, CD3, and CD19. (**B**) Validation of CCL2 depletion in macrophages. Bone marrow-derived cells were isolated from *Ccl2-RFP$^{flox/flox}$* and *LyzM-Cre:Ccl2-RFP$^{flox/flox}$* mice and cultured in DMEM supplemented with 10%

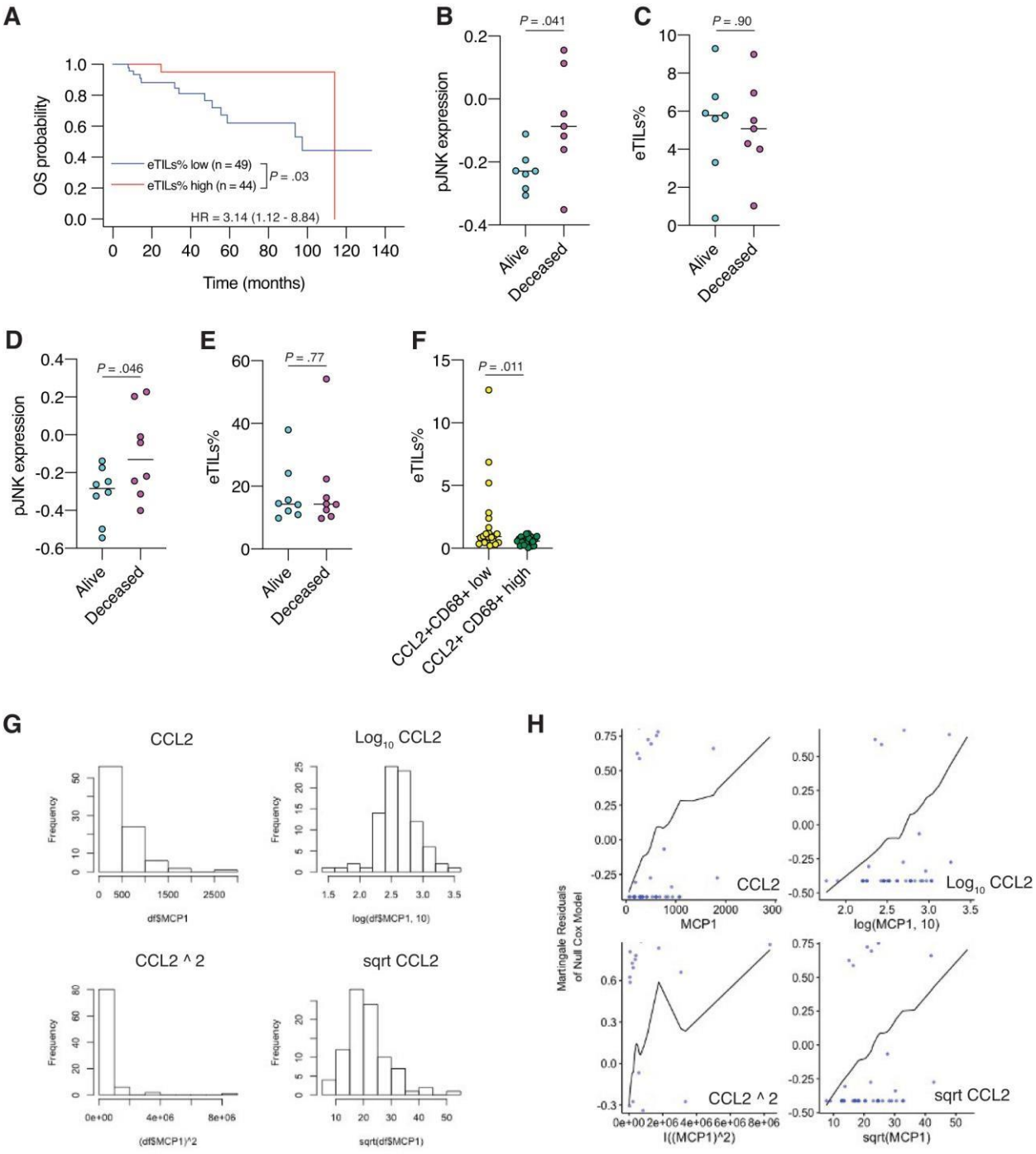
FBS for 6 days. Cells were then subjected to flow cytometry analysis. After excluding debris and dead cells, $CD45^{+}F4/80^{+}$ and $CD45^{+}F4/80^{-}$ cell populations were analyzed for CCL2-RFP expression. the numbers of CCL2-expressing $CD45^{+}F4/80^{+}$ macrophages derived from *LyzM-Cre*:*Ccl2RFP*$^{flox/flox}$ mice were reduced by more than 85% compared with those of *Ccl2-RFP*$^{flox/flox}$ mice, whereas no change was observed in the population of CCL2-expressing $CD45^{+}F4/80^{-}$ cells between *LyzM-Cre*:*Ccl2-RFP*$^{flox/flox}$ mice and *Ccl2-RFP*$^{flox/flox}$ mice. (**C**) Quantification of mean

CCL2 concentration in sera from E0771-inoculated *Ccl2-RFP*$^{flox/flox}$ (n =13) and *LyzM-Cre:Ccl2RFP*$^{flox/flox}$ mice (n =13). The CCL2 levels in sera collected before tumor injection are indicated as a baseline. There was no difference in serum CCL2 concentration between the genotypes before tumor cell inoculation. The CCL2 concentration in serum was significantly reduced in *LyzM-Cre*:*Ccl2-RFP*$^{flox/flox}$ mice versus *Ccl2-RFP*$^{flox/flox}$ mice 3 weeks after E0771 cell inoculation. Error bars represent ± SD. A two-tailed Student *t*-test was used to calculate *P* values.



Supplementary Figure 4. The regulatory role of JNK/C-JUN signaling pathway on CCL2 production in human macrophages. (**A**) Heatmap showing Spearman's correlation coefficient r of expression levels for *CCL2* and representative lineage marker genes for TME components in the TNBC patients from the METABRIC (n = 299) and TCGA (n = 94) cohorts. (**B**) Immunoblotting of phosphorylated JNK, total JNK, and □-actin in M2 THP-1 cells treated with JNK-IN-8 at the indicated concentrations. (**C**) Quantification of the concentration of CCL2 in

CM from M0 THP-1 cells or the indicated TNBC cell lines. The concentrations of CCL2 from each cell line were normalized to 10^6 cells. (**D**) Immunoblotting of JNK1, JNK2, and □-actin in control (Ctrl), JNK1-KO, or JNK2-KO THP-1 cells. (**E**) CD68 expression by human primary macrophages was assessed by flow cytometry, and data are depicted as histogram overlays. The purity of the macrophage populations was confirmed to be more than 95% by flow cytometry. (**F**) Immunoblotting of phosphorylated JNK, total JNK, and □-actin in human primary macrophages treated with JNK-IN-8 at the indicated concentrations. (**G**) Immunoblotting of JNK1, JNK2, and □-actin in human primary macrophages in which JNK1 and JNK2 were silenced by siRNAs. (**H**) Immunoblotting of C-JUN and □-actin in human primary macrophages in which C-JUN was silenced by siRNAs.



I

Multivariate Cox analysis for progression-free and overall survival

Factor	Endpoint	HR	95% CI	*P* value
Log CCL2	Overall survival	2.05	(0.94 - 4.48)	.072
	Progression-free survival	2.08	(1.03 - 4.2)	.041

Supplementary Figure 5. Association of CCL2-expressing TAMs with an immunosuppressive TME, and the correlation between CCL2 serum concentration and prognosis of TNBC patients. (**A**) Kaplan-Meier survival analyses of overall survival (OS) of TNBC patients from the TCGA cohort (n= 93) stratified based on high vs. low eTILs%. The HR with 95% CI is shown on the graph. A log-rank test was used to estimate HR and the significance of the comparison for survival. (**B**) The pJNK expression in alive (n = 7) and deceased (n = 7) patients from the TCGA cohort with low eTILs%. The deceased group expressed higher pJNK levels compared to the alive group. (**C**) The eTILs% of alive and deceased groups with low eTILs%. No significant difference was observed between the two groups. (**D**) The pJNK expression in alive (n = 8) and deceased (n = 8) patients from the TCGA cohort with high eTILs%. The deceased group expressed higher pJNK levels compared to the alive group. (**E**) The eTILs% of the alive and deceased groups with high eTILs%. No significant difference was observed between the two groups. (**F**) The eTILs% in tumors with low (n = 22) and high (n = 21) levels of $CCL2^{+}CD68^{+}$ macrophages. (**G** and **H**) Histograms (**G**) and martingale residuals (**H**) for CCL2 serum concentration (CCL2), logarithmized CCL2 serum concentration ($\log_{10}$ CCL2), squared CCL2 serum concentration (CCL2 ^ 2), and square root of CCL2 serum concentration (sqrt CCL2) from patients with non-metastatic (n = 34) or metastatic TNBC (n = 12). (**I**) Hazard ratios (HRs) and 95% confidence intervals (CIs; represented by the horizontal lines) for overall survival and progression-free survival of TNBC patients (n = 46) according to log-transformed CCL2 level in serum (Log CCL2). A two-tailed Student *t*-test was used to calculate *P* values (**B - F**). A multivariate Cox proportional-hazards model was used to estimate HRs and the significance of the comparison for survival (**I**).