

Supplemental Materials for

Neutrophil–progenitor feedback sustains splenic myelopoiesis and promotes immune evasion in cancer

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Supplemental Methods

In vivo ROS detection

For in vivo ROS measurements, we used the ROS-sensitive chemiluminescent probe L-012 (HY-108537; MCE). L-012 was dissolved in physiological saline (0.9% NaCl solution) and injected via the tail vein (25 mg/kg). Various mouse tissues were harvested 5 min after L-012 injection and imaged on a Xenogen IVIS (PerkinElmer). The images were analyzed with the Living Image software by drawing regions of interest (ROIs) on each tissue to quantify the average radiance (photons s⁻¹ cm⁻² sr⁻¹) emitted.

Extracellular hydrogen peroxide level detection

H₂O₂ levels in bone marrow and spleen lavage fluids, as well as in culture supernatants from various splenic cell populations of tumor-bearing mice, were measured using an Amplex Red assay (KGA7434; keyGen Biotech, Nanjing, China). A standard curve (0–5 μM H₂O₂) was prepared by diluting the H₂O₂ stock solution with the provided reaction buffer. Standards and undiluted samples were added to a 96-well plate. A working solution containing Amplex Red and 0.4 U/mL HRP was added to each well. After incubation at room temperature in the dark for 30 min, fluorescence was measured on a microplate reader (Ex/Em = 530/590 nm).

Cell isolation

Mouse femurs and tibias were collected, and BM cells were flushed with fluorescence-activated cell sorting (FACS) buffer (1% FBS, 2 mM EDTA in PBS) to generate single-cell suspensions. Spleens were homogenized in FACS buffer, passed through a 38-μm filter, and subjected to red blood cell lysis and washing to obtain splenocytes. For isolation of LSK cells, c-Kit⁺ cells were first enriched by magnetic-activated cell sorting (MACS) using anti-CD117 microbeads (130-091-224; Miltenyi Biotec, Germany). Lin^{low/-}Sca1⁺c-Kit^{high} (LSK) cells were then purified using a MoFlo XDP (Beckman Coulter, USA). For isolation of neutrophils, Ly6G⁺ cells were isolated by MACS using a biotin anti-Ly-6G antibody (127603; Biolegend, USA)

and anti-biotin microbeads (130-090-485; Miltenyi Biotec, Germany). For isolation of myeloid descendants derived from cultured HSPCs, Gr-1⁺ cells were isolated by MACS using a biotin anti-Gr-1 antibody (108404; Biolegend, USA) and anti-biotin microbeads (130-090-485; Miltenyi Biotec, Germany). To isolate splenocyte subsets, Ter-119⁺ erythroid cells, CD3⁺/B220⁺ lymphocytes, Ly6G⁺ neutrophils, and Ly6C⁺ monocytes were first enriched by MACS using biotin-conjugated antibodies (BioLegend) and anti-biotin microbeads (130-090-485; Miltenyi Biotec, Germany). Subsequently, CD45⁻Ter-119⁺ Ter-cells, CD3⁺/B220⁺ lymphocytes, CD11b⁺Gr-1^{high}Ly6G⁺Ly6C^{low} neutrophils, and CD45⁺CD11b⁺Gr-1^{int}Ly6G⁻Ly6C^{high} monocytes were sorted to purity by FACS. The purity of sorted populations, assessed by flow cytometry, exceeded 88% after MACS and 93% after FACS.

Flow cytometry (FACS)

Flow cytometry was performed as previously described (1). Detailed information on antibodies and fluorophores is provided in Supplemental Table 2. Cells were stained with antibodies against surface markers in FACS Buffer (1% FBS and 2 mM EDTA in PBS) at 4 °C for 30 min. For flow cytometric analysis of mitochondrial attributes, fresh HSPCs were first stained with surface-marker antibodies, and subsequent mitochondrial dye staining of HSPCs was always performed in a working solution containing 50 µM verapamil (HY-14275; MedChemExpress, USA). For mitochondrial mass measurement, cells were incubated with 100 nM MitoTracker Green (M7514; Thermo Fisher Scientific, USA) in culture medium for 10 min at 37 °C, washed with PBS, and analyzed by flow cytometry. For measurement of the mitochondrial membrane potential, cells were stained with 100 nM tetramethylrhodamine methyl ester (TMRM; T668; Thermo Fisher Scientific, USA) for 30 min at 37 °C, washed with PBS, and analyzed by flow cytometry. For FCCP control experiments, cells were pretreated with 10 µM FCCP for 30 min at 37 °C before TMRM staining and analyzed using the same LSK-gated workflow. For mitochondrial superoxide detection, cells were incubated with 500

nM MitoSOX Red (M36008; Thermo Fisher Scientific, USA) in culture medium for 20 min at 37 °C, washed with PBS, and analyzed by flow cytometry. For intracellular GM-CSF staining, c-Kit⁺ HSPCs were cultured for 6 h at 37 °C in SFEM (09650; STEMCELL, Canada) supplemented with 50 ng/mL recombinant mouse SCF (50487-M08B-100; Sino Biological, Beijing, China) and 5% Leukocyte Activation Cocktail (550583; BD Pharmingen, USA). After stimulation, cells were labeled with antibodies to surface markers, fixed and permeabilized with Fixation/Permeabilization Solution Kit (554714; BD Pharmingen, USA), and then stained intracellularly for GM-CSF. Flow cytometry data were acquired on a CytoFLEX S flow cytometer (Beckman Coulter, USA) and analyzed with FlowJo v10.8. FACS for cell isolation was performed on a MoFlo XDP cell sorter (Beckman Coulter, USA).

Intracellular ROS level detection

After surface antibody staining for flow cytometry, cells were incubated with pre-warmed 2.5 μ M DCFH-DA (HY-D0940; MedChemExpress, USA) at 37 °C for 20 min. Staining was then quenched with RPMI-1640 containing 10% FBS, and cells were kept at 37 °C for 5 min before flow cytometric analysis of DCF fluorescence.

Depletion of Ly6G⁺ neutrophils

To deplete Ly6G⁺ neutrophils, mice received intraperitoneal injections of anti-Ly6G monoclonal antibody (BE0075-1; Bio X Cell, USA) at a dose of 200 μ g per mouse for three consecutive days.

Multiplex immunohistochemical (mIHC) staining and analysis

Chromogenic sequential IHC was conducted with 3.5 μ m FFPE spleen sections from tumor-bearing mice to analyze the spatial distribution of neutrophils and hematopoietic progenitor cells, as well as oxidative stress in progenitor cells. Sections were sequentially stained with c-kit (AF1356; R & D, USA), Ly6G (127602; BioLegend, USA), and CD31 (#77699; Cell Signaling Technology, USA) antibodies for spatial analysis, or with 4-HNE

(ab48506; Abcam, UK), c-kit, and Ly6G antibodies to assess oxidative stress. Detailed information on antibodies is provided in Supplemental Table 3. After deparaffinization, slides were incubated with 0.3% H₂O₂ at room temperature for 10 min to quench endogenous peroxidase activity. Slides then underwent heat-mediated antigen retrieval and were incubated overnight at 4 °C with the respective primary antibodies. After incubation with the species-appropriate secondary antibody, color development was performed using a 3-amino-9-ethylcarbazole (AEC) substrate kit (A2010; Solarbio, Beijing, China). After chromogenic development, the slides were counterstained with hematoxylin (S3301, Dako, Denmark) for 1 min and then scanned using a Leica Aperio Versa 200 (Leica Biosystems, Germany). After scanning, the slides were destained and subjected to the next round of staining. TIFF images of splenic tissue sections were exported from ImageScope (Leica Biosystems, Germany) into HALO (Indica Labs, USA) for image analysis. Based on nuclear centroid segmentation, we quantified nucleus-to-nucleus distances between neutrophils and hematopoietic progenitor cells, and counted the number of neutrophils within 50 µm of each progenitor cell.

Intracellular 4-HNE detection

Intracellular 4-HNE levels were measured using a 4-HNE ELISA kit (STA-838; Cell Biolabs, USA). LSK cells were sorted from the bone marrow and spleen of tumor-bearing mice, along with HSPCs from the co-culture model. Briefly, 50 µL of cell lysates or HNE-BSA standards were added to a 96-well plate and incubated at room temperature for 10 min. Next, 50 µL of anti-HNE antibody was added to each well and incubated for 1 h at room temperature, followed by three washes. Diluted HRP-conjugated secondary antibody was then added, incubated for 1 h at room temperature, and washed three times. After adding the substrate solution and incubating for 30 min, 100 µL of stop solution was added to each well. Absorbance was measured at 450 nm using a microplate reader.

Organ-targeted drug delivery

Micro-osmotic pump systems (1002W; RWD, Shenzhen, China and 0007700; Alzet, USA) were loaded with 500 µg of Mdivi-1 (HY-15886; MedChemExpress, USA), 2 mg of NAC (HY-B0215; MedChemExpress, USA), 100 µg of HLZ (H1753; Sigma-Aldrich, USA), or vehicle (saline solution containing 10% DMSO). The pumps were preconditioned overnight at 37 °C in sterile saline to ensure steady delivery upon implantation. Micro-osmotic pumps preloaded with jugular catheters were implanted subcutaneously in the dorsal neck region of C57BL/6 mice, with the catheter tip inserted into the subcapsular space of the spleen or the orthotopic tumor site. The infusion rate was 0.25 µL/h. All compounds were prepared in saline with 10% DMSO, and control animals received micro-osmotic pumps filled with the same 10% DMSO saline vehicle.

Single-cell RNA sequencing of HSPCs

For scRNA-seq of splenic HSPCs, mice bearing orthotopic Hepa1-6 tumors received splenic-targeted drug delivery (vehicle or NAC) using the micro-osmotic pump systems as described above. Live single-cell suspensions from BM and spleen were prepared, LSK cells were sorted by FACS, washed, counted, and immediately processed for library preparation. Single-cell 3' libraries were prepared on the Chromium Controller (10x Genomics, USA) using Single Cell 3' v3 chemistry (10x Genomics, USA) and performed by Berry Genomics Co. Ltd (Guangzhou, China), following the manufacturer's protocol. Libraries were sequenced on an Illumina NovaSeq 6000 platform, achieving >30% sequencing saturation as estimated by *Cell Ranger*. Raw sequencing data were processed with *Cell Ranger count* pipeline (v7.0.0) against the mouse reference genome mm10 (refdata-gex-mm10-2020-A), with intronic reads included (include-introns=true).

scRNA-seq processing, quality control, integration, and analysis

Raw 10x gene-count matrices (*raw_feature_bc_matrix*) were processed to remove ambient RNA using *CellBender* (v0.3.0), then analyzed in *R* (v4.3.3). QC metrics were computed per cell; cells were retained with ≥ 500 detected genes, $\leq 20\%$ mitochondrial gene counts, $\leq 1\%$ hemoglobin gene counts, and were not predicted as doublets. Doublets were identified with *scDblFinder* (1.14.0). Normalization and multi-sample integration were performed with *Seurat* (v5.1.0) using *SCTransform* v2 method (regularized negative binomial regression), with regression of mitochondrial percentage and the difference between S and G2M scores (S.Score - G2M.Score). Integration used SCT features and Reciprocal PCA (RPCA) anchors. PCA/UMAP were computed on scaled integrated data after excluding histone, mitochondrial, hemoglobin, ribosomal protein, and repetitive-element-associated transcripts. Clustering was performed with graph-based clustering (resolution 0.2), and cluster identities were assigned using canonical marker genes and differential expression. For pathway interpretation, gene set enrichment analysis (GSEA) was conducted with *clusterProfiler* (v4.8.1) (FDR < 0.05). Signature activities were scored with UCell and visualized on UMAPs. Differentiation trajectories were inferred with *Slingshot* algorithm (v2.8.0) (root: HSC). The analysis code is available at https://github.com/ChongEdwardWu/JCI_2026_Neutro-HSPC-feedback, and the corresponding result tables are provided in Supplemental Table 4.

Confocal and super-resolution structured illumination microscopy

For mitochondrial morphology analysis using mitochondrial fluorescent dye, purified LSK cells from mouse bone marrow and spleen, as well as HSPCs from the neutrophil co-culture model (see below), were stained to visualize mitochondria. Cells were incubated at 37 °C for 50 min in staining buffer containing MitoTracker (green) (M7514; Thermo Fisher Scientific, USA) to label mitochondria and Hoechst 33342 (HY-15559; MedChemExpress, USA) to label nuclei, with 50 μ M Verapamil (HY-14275; MedChemExpress, USA) in the staining solution to

inhibit dye efflux. Following staining, live cells were immediately imaged by super-resolution structured illumination microscopy (HIS-SIM; CSR-BIOTECH, Guangzhou, China) or Confocal laser scanning microscopy (TCS SP8X, Leica, Germany). Mitochondrial morphology was quantified by skeletonizing the fluorescence images and measuring mitochondrial branch length using the AnalyzeSkeleton plugin in ImageJ (National Institutes of Health NIH, USA).

To examine the subcellular distribution of FOXO1, we assessed the nuclear-to-cytoplasmic ratio of the protein by immunofluorescence. Purified HSPCs (5×10^4 cells per slide) were deposited onto glass slides by cytocentrifuge (Cytopro 7620, Wescor, USA) and fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature. After three washes with PBS, the samples were blocked at room temperature for 1 hour with 5% BSA and 0.03% Triton X-100. Subsequently, the slides were washed with TBST. Then, cells were incubated with FOXO1 antibodies (2880S; Cell Signaling Technology, USA) followed by fluorescence-conjugated secondary antibody: Donkey anti-Rabbit IgG (H+L), Highly cross-adsorbed secondary antibody (A21206; ThermoFisher, USA). Nuclei were stained with DAPI (D1306; Thermo Fisher Scientific, USA). Confocal laser scanning microscopy (TCS SP8X, Leica, Germany) was performed to visualize FOXO1 staining, and the nuclear-to-cytoplasmic ratio of FOXO1 was quantified using ImageJ.

Transmission electron microscopy

Pellets of 1.5×10^5 purified LSK cells were fixed with a fixation solution containing 3% glutaraldehyde and 2% paraformaldehyde at 4 °C for 24 h, then post-fixed in 1% osmium tetroxide. Samples were dehydrated through sequential washes in 50%, 70%, 90%, and 95% ethanol, followed by acetone, and embedded in low-gelling-temperature agarose (EM0300; Sigma-Aldrich, USA). Ultrathin sections (100 nm) were collected on 200-mesh copper grids, stained with lead citrate, and carbon-coated. The section grids were examined on an electron microscope (JEM 1400; JEOL, Japan). Mitochondrial length was defined as the long-axis

length and quantified in ImageJ. The mean mitochondrial length for each sample was determined by measuring individual mitochondria within single cells and aggregating data from at least 50 cells.

Seahorse metabolic assays

Freshly isolated LSK cells (3.5×10^4 cells per well) were seeded onto Cell-Tak-coated Seahorse culture plates (354240; Corning, USA) in Seahorse XF assay medium. Mitochondrial respiration was assessed using the Seahorse XF Cell Mito Stress Test Kit (103015-100; Agilent, USA) on a Seahorse XFe24 Extracellular Flux Analyzer (Agilent Technologies, USA). Oxygen consumption rate (OCR) was measured at baseline and after sequential injections of 2 μ M oligomycin (ATP synthase inhibitor), 1.5 μ M FCCP (uncoupler), and a mix of 0.5 μ M antimycin A plus 0.5 μ M rotenone (complex III and I inhibitors, respectively).

Immunoblotting

For immunoblotting assays, fresh purified HSPCs were washed in RPMI-1640 containing 0.1% phenylmethylsulfonyl fluoride (A100754; Sangon Biotech, Shanghai, China), pelleted, and lysed in lysis buffer at $\sim 1,000$ cells per 1 μ L. The lysis buffer consisted of 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate, 10% (w/v) glycerol, 50 mM dithiothreitol, and 0.01% (w/v) bromophenol blue in ultrapure water. Lysates were centrifuged at 12,000g for 8 min, and supernatants were collected. Samples were then run on 10% SDS-PAGE gels. Separated proteins were then transferred to PVDF membranes, which were blocked for 2.5 h at room temperature in 5% w/v milk or 5% w/v BSA, then probed with the indicated primary antibodies followed by appropriate HRP-conjugated secondary antibodies. Signals were detected with the SuperSignal West Pico chemiluminescent substrate (34580, Thermo Fisher Scientific, USA) and imaged. Primary antibodies included a mitochondrial dynamics antibody sampler kit (Mitochondrial Dynamics Antibody Sampler Kit II; 74792; Cell Signaling Technology, USA), FOXO1 antibodies (2880S; Cell Signaling Technology, USA), JNK

antibodies (9252S; Cell Signaling Technology, USA), p-JNK antibodies (4668S; Cell Signaling Technology, USA), AKT antibodies (4691S; Cell Signaling Technology, USA), p-AKT antibodies (4060S; Cell Signaling Technology, USA), STING antibodies (13647S; Cell Signaling Technology, USA), p-STING antibodies (72971S; Cell Signaling Technology, USA), TBK1 antibodies (3504S; Cell Signaling Technology, USA), p-TBK1 antibodies (5483S; Cell Signaling Technology, USA), p65 antibodies (8242S; Cell Signaling Technology, USA), p-p65 antibodies (3033S; Cell Signaling Technology, USA), I κ B α antibodies (4812S; Cell Signaling Technology, USA), p-I κ B α antibodies (2859T; Cell Signaling Technology, USA), β -actin antibodies (BM0627; BOSTER, Wuhan, China). Secondary antibodies included Anti-mouse IgG, HRP-linked Antibody (7076S; Cell Signaling Technology, USA), Anti-rabbit IgG, HRP-linked Antibody (7074S; Cell Signaling Technology, USA).

RT-PCR

The total RNA of HSPCs was extracted using TRIzol reagent (15596018; Thermo Fisher Scientific, USA) according to the manufacturer's instructions. cDNA was synthesized using the All-in-One RT Master Mix (G492; Applied Biological Material, Canada). Quantitative PCR was performed with SYBR Green qPCR Master Mix (B21203; Bimake, USA) on a LightCycler 480 system (05015243001; Roche, Switzerland). All reactions were run in triplicate.

Primers for *Dnm1l*: Forward (5'-3') GCGAACCTTAGAATCTGTGGACC; Reverse (5'-3') CAGGCACAAATAAAGCAGGACGG

Murine HSPCs in vitro cultures

LSK cells were cultured in Serum-Free Expansion Medium (SFEM) (09650; STEMCELL, Canada) supplemented with 20 ng/mL SCF (50487-M08B-100; Sino Biological, Beijing, China), 20 ng/mL TPO (50146-M08H-100; Sino Biological, Beijing, China), and 100 ng/mL Flt3-L (51113-M02H-100; Sino Biological, Beijing, China). For co-culture experiments, 5×10^4 cells/mL naïve BM LSK cells were co-cultured with 5×10^5 cells/mL neutrophils. To

examine mitochondrial morphology and function, LSK–neutrophil co-cultures were maintained for 16 h before analysis. To induce myeloid differentiation of progenitors, LSK cells were first cultured alone for 48 h and then co-cultured with neutrophils for an additional 48 h. LSK cells were also stimulated with splenic stromal cell supernatant (SSN) and L-buthionine-(S,R)-sulfoximine (BSO; HY-106376A; MedChemExpress, USA) in vitro. SSNs were harvested from stromal cells (CD45⁻ non-immune cells) cultured for 48 h at a density of 5×10^6 cells/mL in RPMI-1640 (Cat# 31800089, Thermo Fisher Scientific, USA) supplemented with 10% FBS. SSN was used at a final concentration of 5% to stimulate the LSK cells, and BSO at a final concentration of 150 nM. In in vitro experiments, NAC was used at a working concentration of 1 mM, the mitochondrial fission inhibitor Mdivi-1 at 5 μ M, and the FOXO1 inhibitor AS1842856 at 2 μ M. Where indicated, the JNK inhibitor SP600125 was added at 2 μ g/mL (9.08 μ M).

Small guide RNA design

Small guide RNAs (sgRNAs) were designed using SYNTHIGO's online tool (<https://design.synthego.com/#/>).

The sequence for sg-*Foxo1* was CACCTGGGGCGCTTCGGCCA. The sequences for sg-*Dnm1l* were TATTTGCAGCAGTGACGGCG and AGCAGTGACGGCGAGGATAA.

Retrovirus production

To edit Cas9-expressing LSK cells, we constructed U6-driven sgRNA expression cassettes targeting the murine *Foxo1* or *Dnm1l* gene and inserted them into the MSCV-IRES-Thy1.1 DEST vector. For the sgRNA control (sg-Ctrl), the MSCV-IRES-Thy1.1 DEST vector containing only the sgRNA scaffold under U6 promoter control was employed. HEK293T cells were transfected with the constructed vectors together with pCL-Eco using polyethylenimine (23966-1; Polysciences, USA). Transfections were performed in DMEM.

LSK cell transduction

For the gene-editing experiments using the CRISPR-Cas9 system, 1×10^5 - 2×10^5 native LSK cells were purified from CAG-Cas9-tdTomato-expressing mice. LSK cells were maintained in SFEM supplemented with 20 ng/mL SCF and 100 ng/mL TPO. After 36 h, LSK cells were transduced with retrovirus on RetroNectin-coated 24-well plates ($4 \mu\text{g}/\text{cm}^2$; Takara, Japan). Thirty-six hours later, Thy1.1⁺ cells were FACS-sorted and cultured in SFEM (20 ng/mL SCF, 100 ng/mL TPO) for an additional 24 h prior to harvest.

CFU-C assay

The CFU-C assays were conducted in methylcellulose-based complete medium (MethoCult GF M3434; STEMCELL Technologies, Canada) following the manufacturer's instructions. Colonies were enumerated and morphologically classified after 14 days of culture. All cultures were maintained at 37 °C in a humidified incubator with 5% CO₂.

T cell proliferation assay

For the murine T cell proliferation assay, naïve splenocytes were labeled with 2 μM CFSE (C34554; Thermo Fisher Scientific, USA) and cultured in RPMI-1640 (31800089; Thermo Fisher Scientific, USA) supplemented with 10% FBS, 10 ng/mL recombinant IL-2 (202-IL-500; R & D, USA), 1 $\mu\text{g}/\text{mL}$ anti-CD3 (16-0031; Thermo Fisher Scientific, USA), and 3 $\mu\text{g}/\text{mL}$ anti-CD28 (16-0281; Thermo Fisher Scientific, USA). Labeled splenocytes were co-cultured, at the indicated ratios, with either freshly isolated neutrophil-like cells (CD11b⁺Gr-1^{high}Ly6G⁺Ly6C^{low}), monocyte-like cells (CD11b⁺Gr-1^{high}Ly6G⁻Ly6C⁺) or myeloid descendants derived from cultured HSPCs (CD11b⁺Gr-1⁺). After 84 h, proliferation of CD8⁺ T cells was evaluated by flow cytometry. The division index, representing the average number of cell divisions per cell in the original population, was calculated using FlowJo and normalized to the T cell-only group in each experiment.

Intrasplenic co-transfer of LSK cells and neutrophils

For the single adoptive transfer experiment, a total of 1×10^5 CD45.1⁺ BM LSK cells from healthy mice were mixed with 1×10^6 CD45.2⁺ splenic neutrophils isolated from healthy or tumor-bearing WT or *Cybb*^{-/-} mice and delivered by direct intrasplenic injection using a syringe into the spleen of CD45.2⁺ healthy recipient mice. The progeny derived from the transferred hematopoietic progenitor cells, as well as the splenic immune microenvironment of recipient mice, were subsequently analyzed.

For the sequential adoptive transfer experiment, following the initial transfer of CD45.2⁺ cells, an additional 1×10^5 CD45.1⁺ BM LSK cells were administered into the same recipient mice 6 days later. The progeny derived from this second transfer of progenitor cells and the splenic immune microenvironment were then evaluated.

Recipient mice were provided with antibiotic-containing drinking water (pH 2.5–3.0; 0.5 mg/mL neomycin and 0.17 mg/mL enrofloxacin).

Isolation of human splenic HSPCs

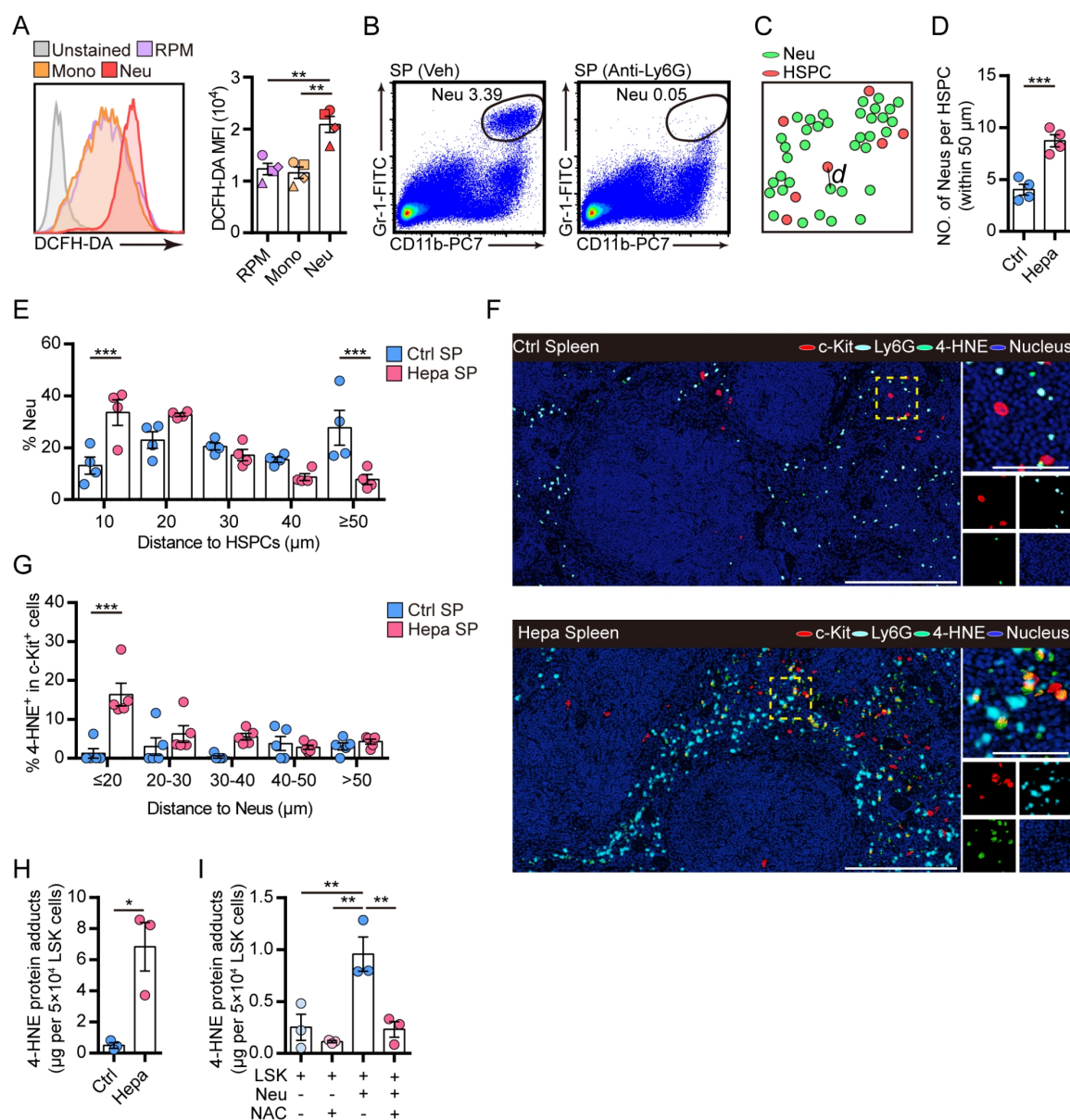
Human splenic tissue was minced into small pieces using fine scissors and then digested in RPMI-1640 supplemented with collagenase I (C0130; Sigma-Aldrich, USA), collagenase IV (C5138; Sigma-Aldrich, USA), DNase I (D5025; Sigma-Aldrich, USA), hyaluronidase (H3884; Sigma-Aldrich, USA), and 20% FBS at 37 °C with agitation at 750 rpm for 40 min. The cell suspension was passed through a 38- μm filter, followed by red blood cell lysis, washing, and resuspension in RPMI-1640. CD133⁺ splenic HSPCs were enriched by MACS using CD133 microbeads (130-100-857; Miltenyi, Germany) according to the manufacturer's instructions. After FSC/SSC-based debris exclusion, the purity of CD45⁺Lin⁻CD34⁺ cells within the MACS-enriched CD133⁺ preparations exceeded 90%. The MACS-enriched CD133⁺ cells were then used for mitochondrial imaging. For mitochondrial morphology analysis, cells were stained with MitoTracker Green and Hoechst 33342 at 37 °C for 45 min, followed by

assessment of mitochondrial morphology.

Human CB-HSPC co-culture assay

Human CD34⁺ CB-HSPC culture was performed as previously described (Wu et al, *JCI*, 2018, Ref.(2)); (Wu et al, *PNAS*, 2014, Ref.(3)). Briefly, CD34⁺ cells were isolated from fresh human umbilical cord blood by MACS. To generate CB-HSPC-derived neutrophils, on day 7 of CD34⁺ HSPC expansion, 5×10^5 CD34⁺ cells were seeded into 24-well plates and cultured in RPMI-1640 supplemented with 10% FBS, 40 ng/mL GM-CSF (215-GM; R & D, USA), and 40 ng/mL G-CSF (10007-HNCH; Sino Biological, Beijing, China) for 4 days. CD15⁺ cells were then purified by MACS and labeled with 2.5 μ M CFSE. Next, we co-cultured CD34⁺ CB-HSPCs with CD15⁺ CB-HSPC-derived neutrophils for 4 days and analyzed the CFSE⁻ cell population. For FOXO1 localization and DRP1 expression analyses, CD34⁺ HSPCs were recovered after co-culture and processed for immunofluorescence or immunoblotting as indicated. To assess the instructive effect of CD15⁺ cell-derived ROS on CD34⁺ HSPCs, we sorted CFSE⁻ Lin⁻ human HSPCs after four days of co-culture and cultured them for an additional four days in the presence or absence of NAC and HLZ, followed by analysis of the differentiated progeny. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂.

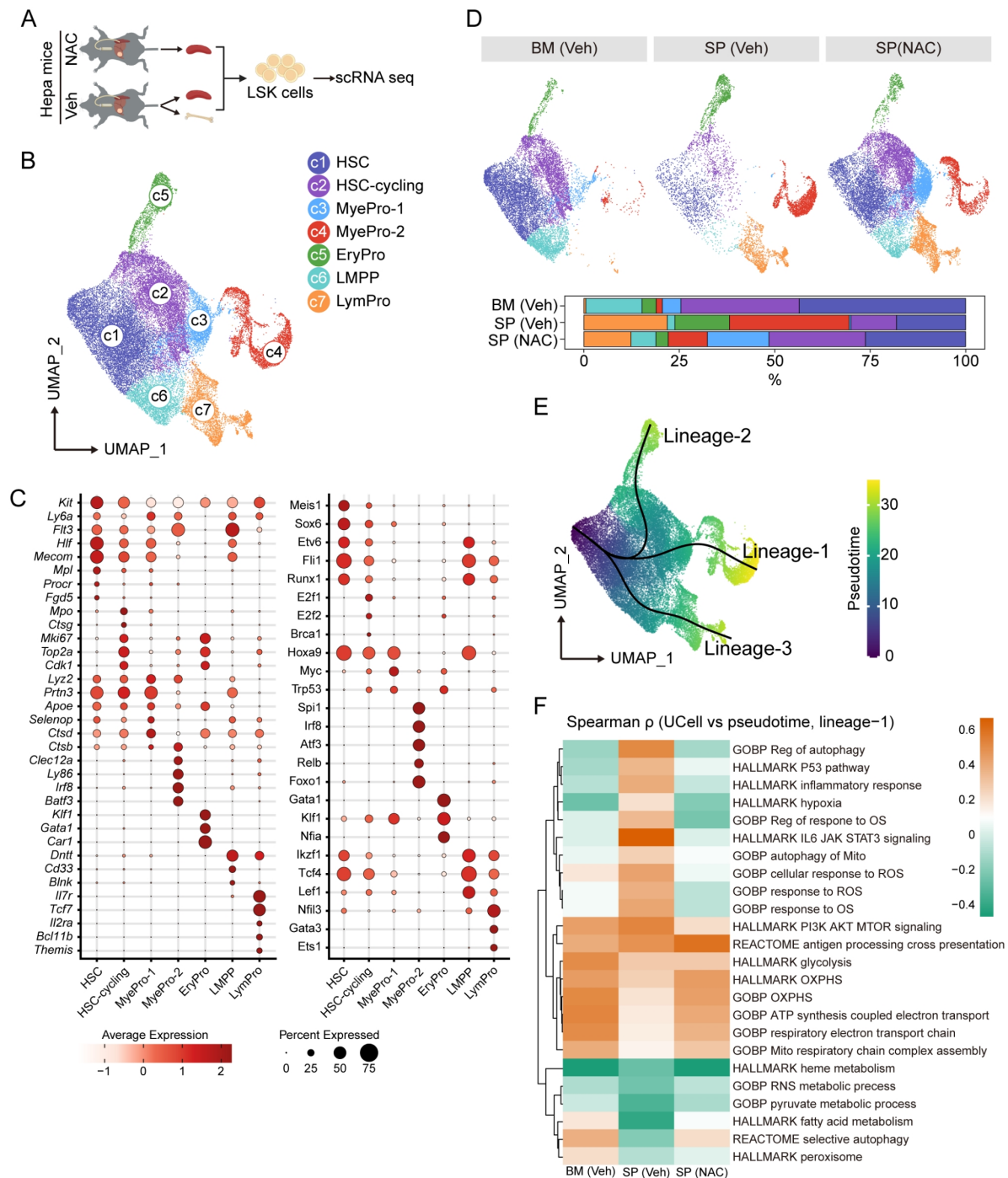
Supplemental Figures



Supplemental Figure 1. Neutrophil-derived oxidative stress dominates the splenic niche in tumor-bearing mice.

(A) Intracellular ROS levels in red pulp macrophages (RPM), monocytes (Mono) and neutrophils (Neu) isolated from SP of Hepa mice, measured by DCFH-DA flow cytometry ($n = 4$ per group); histogram (left) and MFI quantification (right). (B) Depletion efficiency of neutrophils in the spleen of tumor-bearing mice. Data are representative of $n = 3-4$ donors. (C) Schematic of nearest-neighbor analysis illustrating measurement of the shortest distance (d)

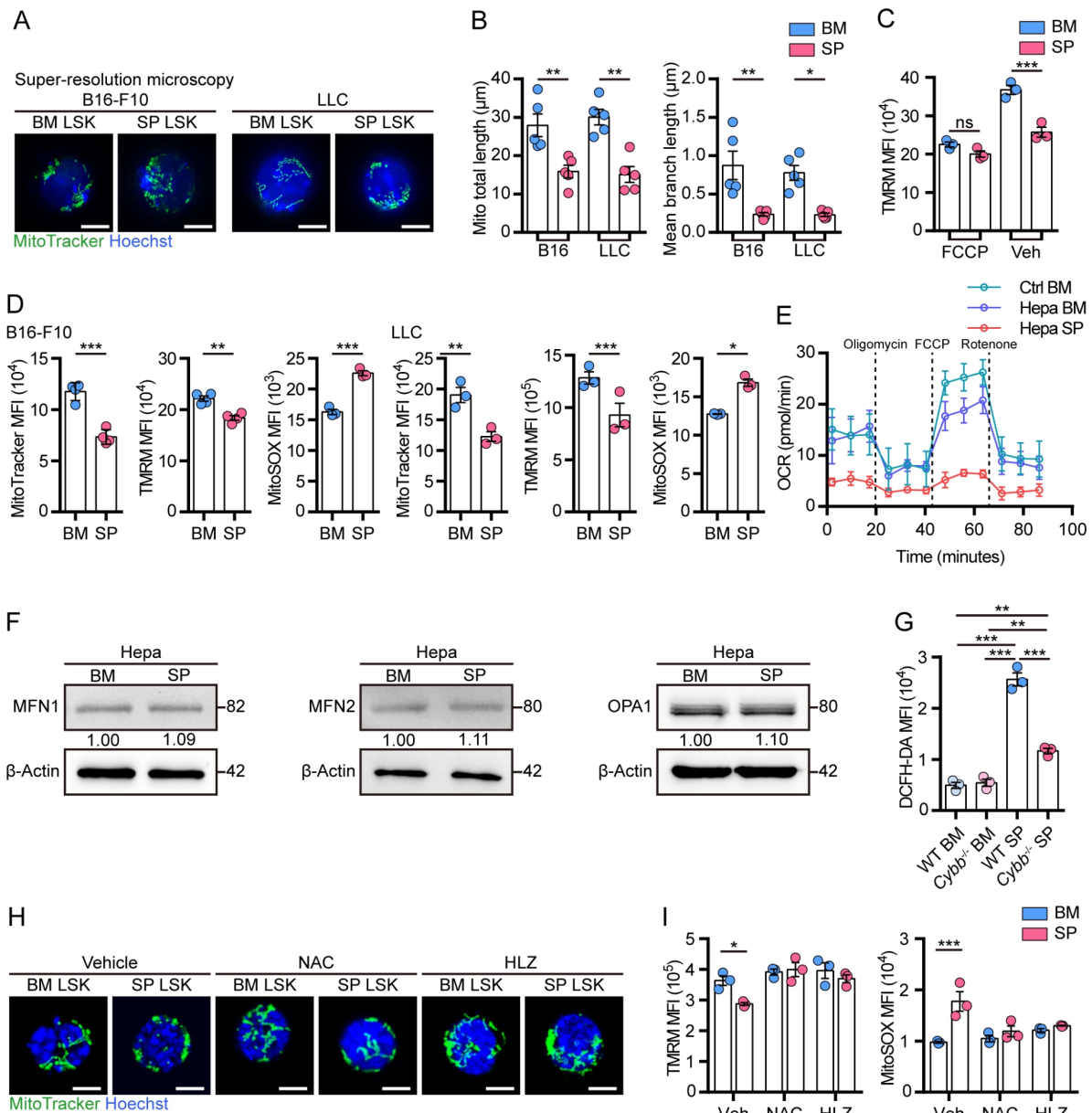
from each Ly6G⁺ neutrophil to the closest c-Kit⁺ HSPC in spleen sections, calculated based on nuclear centroids. **(D)** Quantification of the number of Ly6G⁺ neutrophils within a 50 μ m radius of each c-Kit⁺ HSPC in spleen sections from Ctrl and Hepa mice ($n = 4$ per group). **(E)** Distribution of neutrophil-to-HSPC distances in splenic red pulp, binned into defined distance ranges, comparing Ctrl and Hepa mice ($n = 4$ mice per group). **(F)** Multiplex immunohistochemistry (mIHC) of spleen sections from Ctrl and Hepa mice, stained for hematopoietic progenitor cells (HSPC-enriched c-Kit⁺ cells), neutrophils (Neu; Ly6G⁺), and 4-HNE (4-HNE⁺). Scale bars, 200 μ m in the overview images and 50 μ m in the zoomed in images. **(G)** Percentage of 4-HNE⁺ HSPCs located at the indicated distances from the nearest neutrophils (Neus) in the spleens of Ctrl and Hepa mice ($n = 5$ per group). **(H)** 4-HNE levels in LSK cells from the spleens of Ctrl and Hepa mice ($n = 3$ per group). **(I)** 4-HNE levels in bone marrow LSK cells from healthy mice after co-culture with splenic neutrophils from tumor-bearing mice, in the presence or absence of NAC (1 mM) ($n = 3$ per group). Data are mean $\pm$ SEM. Statistics: Student's t test (**D, H**); one-way ANOVA corrected by Tukey's (**A**), Dunnett's (**I**) post-hoc test; two-way ANOVA corrected by Sidak's (**E, G**) post-hoc test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



Supplemental Figure 2. Splenic LSK cells under oxidative stress exhibit a pronounced myeloid differentiation bias and mitochondrial stress characteristics.

(A) Scheme of the scRNA-seq analysis of BM and splenic LSK cells from Hepa mice and splenic LSK cells from NAC-treated (spleen-targeted drug delivery) Hepa mice. Veh, vehicle; NAC, N-acetylcysteine. (B) UMAP projection of scRNA-seq profiles from sorted LSK cells,

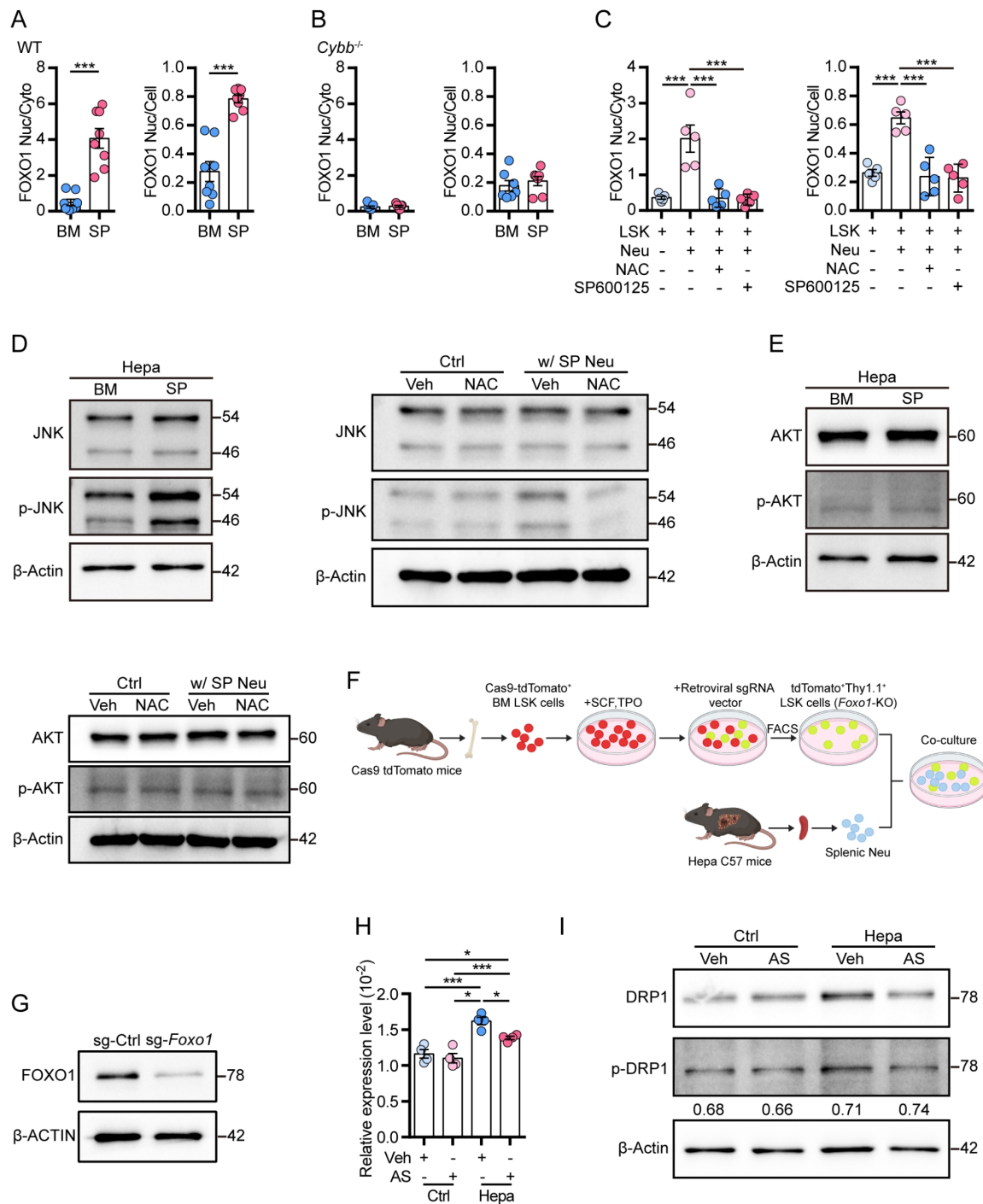
with clusters annotated by cell types. HSC, hematopoietic stem and multipotent cell; MyePro, myeloid-biased progenitor; EryPro, erythroid progenitor; LMPP, lymphoid-primed multipotent progenitor; LymPro, lymphoid-biased progenitor. **(C)** Left: dot-plot of marker genes across LSK clusters resolved in **(B)**. Dot color indicates average SCT-normalized expression, and dot size indicates the percentage of cells with detectable expression (non-zero counts). Right: Dot-plot of transcription factor regulon activity for the same LSK clusters as in **(C-left)**. Dot color indicates mean binarized regulon activity score (AUCell scores converted into binary ON/OFF states), and dot size indicates the fraction of cells in each cluster with the regulon recognized “ON”. HSC, hematopoietic stem and multipotent cell; MyePro, myeloid-biased progenitor; EryPro, erythroid progenitor; LMPP, lymphoid-primed multipotent progenitor; LymPro, lymphoid-biased progenitor; TF, transcription factor; AUCell, area-under-the-curve-based regulon activity scoring. **(D)** Comparison of LSK cluster compositions between groups. **(E)** Trajectory analysis and pseudotime of LSK cell differentiation. Lineage-1, myeloid; lineage-2, erythroid; lineage-3, lymphoid. **(F)** Spearman’s ρ correlation analysis between UCell gene-set module scores and pseudotime of LSK differentiation along Lineage-1. OS, oxidative stress; Mito, mitochondria.



Supplemental Figure 3. Mitochondrial morphology of LSK cells across tumor models and ROS modulation in the splenic niche.

(A) Super-resolution microscopy images of purified BM and SP LSK cells from B16- F10 and LLC tumor-bearing mice, stained with MitoTracker Green (mitochondria, green) and Hoechst (nuclei, blue). Scale bars, 5 μ m. (B) Quantification of total mitochondrial length and mean branch length of BM and SP LSK cells from B16- F10 and LLC tumor-bearing mice ($n = 5$ per group). (C) Quantification of TMRM mean fluorescence intensity (MFI) in paired BM and SP LSK cells from Hepa mice under vehicle (Veh)- and carbonyl cyanide-4-

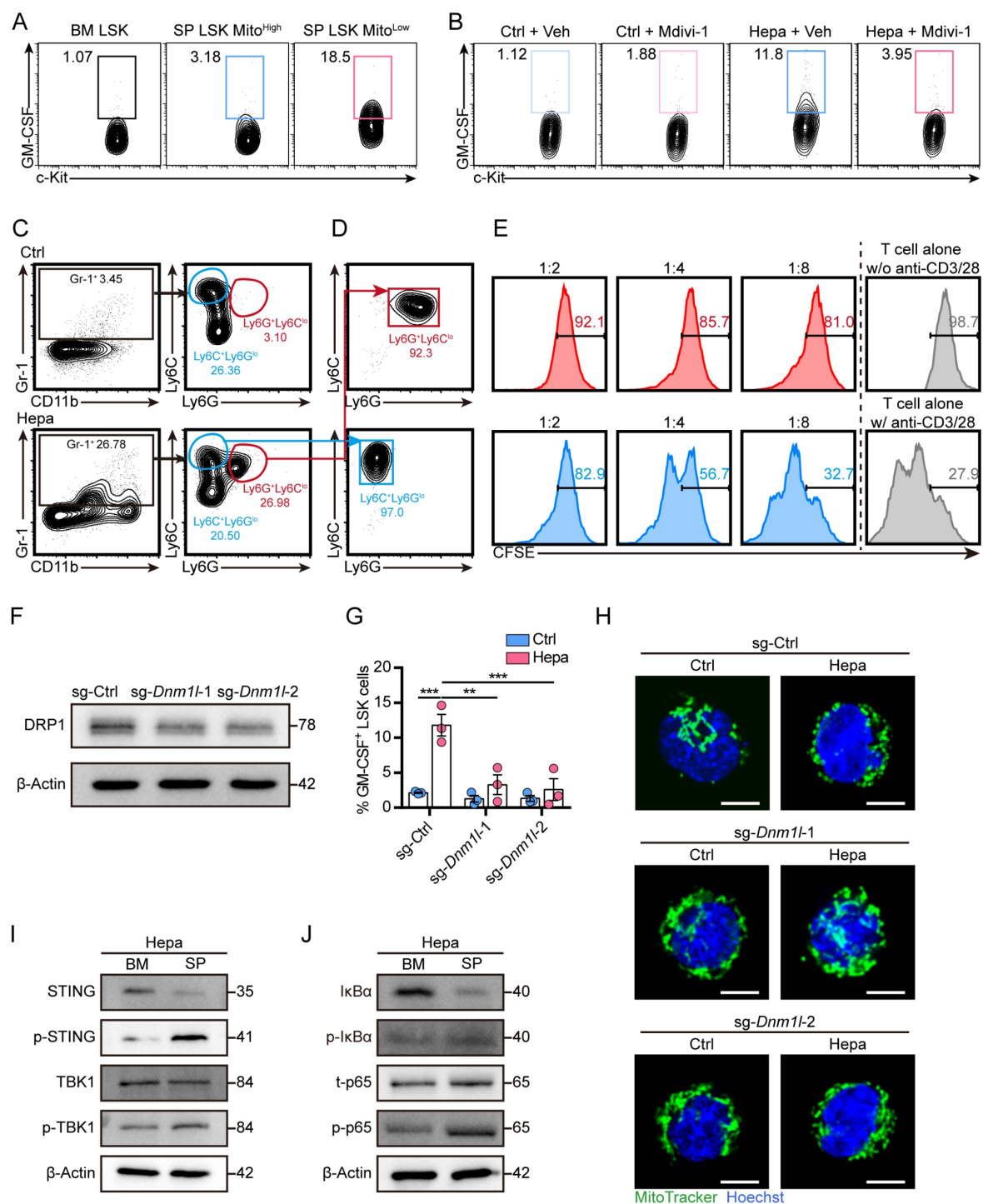
(trifluoromethoxy)phenylhydrazone (FCCP)-treated conditions ($n = 3$ per group). **(D)** Flow cytometric assessment of mitochondrial mass (MitoTracker Green), membrane potential (TMRM), and mitochondrial ROS (MitoSOX) in paired BM and SP LSK cells from B16- F10 and LLC mice ($n = 3$ -4 per group). **(E)** Seahorse oxygen-consumption rate (OCR) traces of control BM, Hepa BM, and Hepa SP LSK cells. ($n = 3$ per group). Data are from one experiment with 3.5×10^4 cells per sample. **(F)** Immunoblotting of MFN1, MFN2, and OPA1 in BM and SP LSK cells from Hepa mice ($n = 3$ samples per group). **(G)** Intracellular ROS levels in neutrophils from BM and SP of WT and *Cybb*^{-/-} Hepa mice, measured using DCFH-DA. ($n = 3$ mice per group). **(H-I)** Pharmacologic ROS scavenging restores mitochondrial morphology and function in splenic LSK cells ($n = 3$ per group). NAC, N-acetylcysteine; HLZ, hydralazine. Confocal images of BM and SP LSK cells from Hepa mice treated with vehicle, NAC, or HLZ **(H)**; scale bars, 5 μ m. Quantification of mitochondrial membrane potential (TMRM) and mitochondrial ROS (MitoSOX) by flow cytometry **(I)**. Data are mean $\pm$ SEM. Statistics: Student's t test **(D)**; one-way ANOVA corrected by Tukey's **(B, C, G)** post-hoc test; two-way ANOVA corrected by Dunnett's **(I)** post-hoc test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



Supplemental Figure 4. Neutrophil-derived ROS induce mitochondrial fragmentation in HSPCs.

(A) Quantification of the nuclear vs. cytoplasmic (Nuc/Cyto) and nuclear vs. total cellular (Nuc/Cell) FOXO1 ratios in BM and SP LSK cells from WT Hepa mice ($n = 8$ per group). (B) Quantification of FOXO1 localization in BM and SP LSK cells from *Cybb*^{-/-} Hepa mice ($n = 7$

per group). **(C)** Quantification of FOXO1 localization in BM LSK cells from healthy mice co-cultured with splenic neutrophils isolated from Hepa mice, in the absence or presence of the ROS scavenger NAC or JNK inhibitor SP600125 (2 μ g/mL) ($n = 5$ per group). **(D)** Left, immunoblotting of JNK and p-JNK in BM and SP LSK cells from Hepa mice ($n = 3$ samples per group). Right, immunoblotting of JNK and p-JNK in BM LSK cells from healthy mice co-cultured with splenic neutrophils isolated from Hepa mice, in the absence or presence of the ROS scavenger NAC ($n = 3$ samples per group). **(E)** Up, immunoblotting of AKT and p-AKT in BM and SP LSK cells from Hepa mice ($n = 3$ samples per group). Down, immunoblotting of AKT and p-AKT in BM LSK cells from healthy mice co-cultured with splenic neutrophils isolated from Hepa mice, in the absence or presence of the ROS scavenger NAC ($n = 3$ samples per group). **(F)** Schematic of the CRISPR/Cas9 *Foxo1* knock-out strategy. tdTomato⁺ LSKs from Cas9 mice were expanded in vitro, transduced with sg-*Foxo1* or control retrovirus, sorted (tdTomato⁺Thy1.1⁺), and co-cultured with splenic neutrophils from Hepa mice. **(G)** Immunoblotting of FOXO1 in sg-ctrl and sg-*Foxo1* LSK cells. **(H-I)** *Dnm1l* mRNA expression levels **(H)** and immunoblotting of DRP1 and p-DRP1 **(I)** were detected in BM LSK cells from healthy mice cultured in vitro with splenic stromal supernatants to mimic tumor-free (Ctrl) or tumor-bearing (Hepa) spleen-conditioned environments (see Methods for splenic stromal supernatant) with vehicle (Veh) or the FOXO1 inhibitor AS1842856 (2 μ M). Numbers under the total DRP1 blot indicate the p-DRP1 Ser616/total DRP1 ratio. ($n = 3$ -4 samples per group). Data are mean $\pm$ SEM. Statistics: Student's t test (**A, B**); one-way ANOVA corrected by Tukey's (**C, H**) post-hoc test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

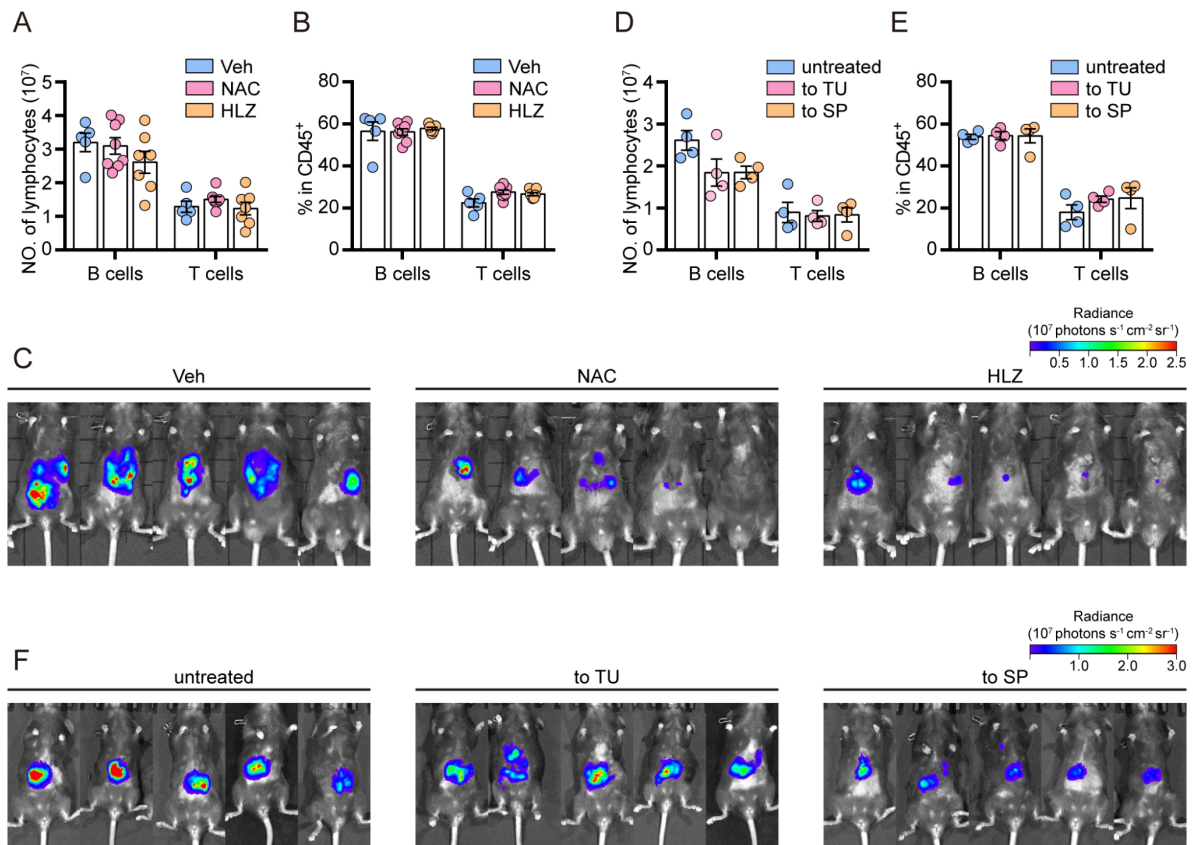


Supplemental Figure 5. GM-CSF induction, *Dnm1l* perturbation, and STING-NF-κB activation in splenic HSPCs.

(A) Percentage of GM-CSF⁺ cells in the indicated LSK subsets, determined by flow cytometry.

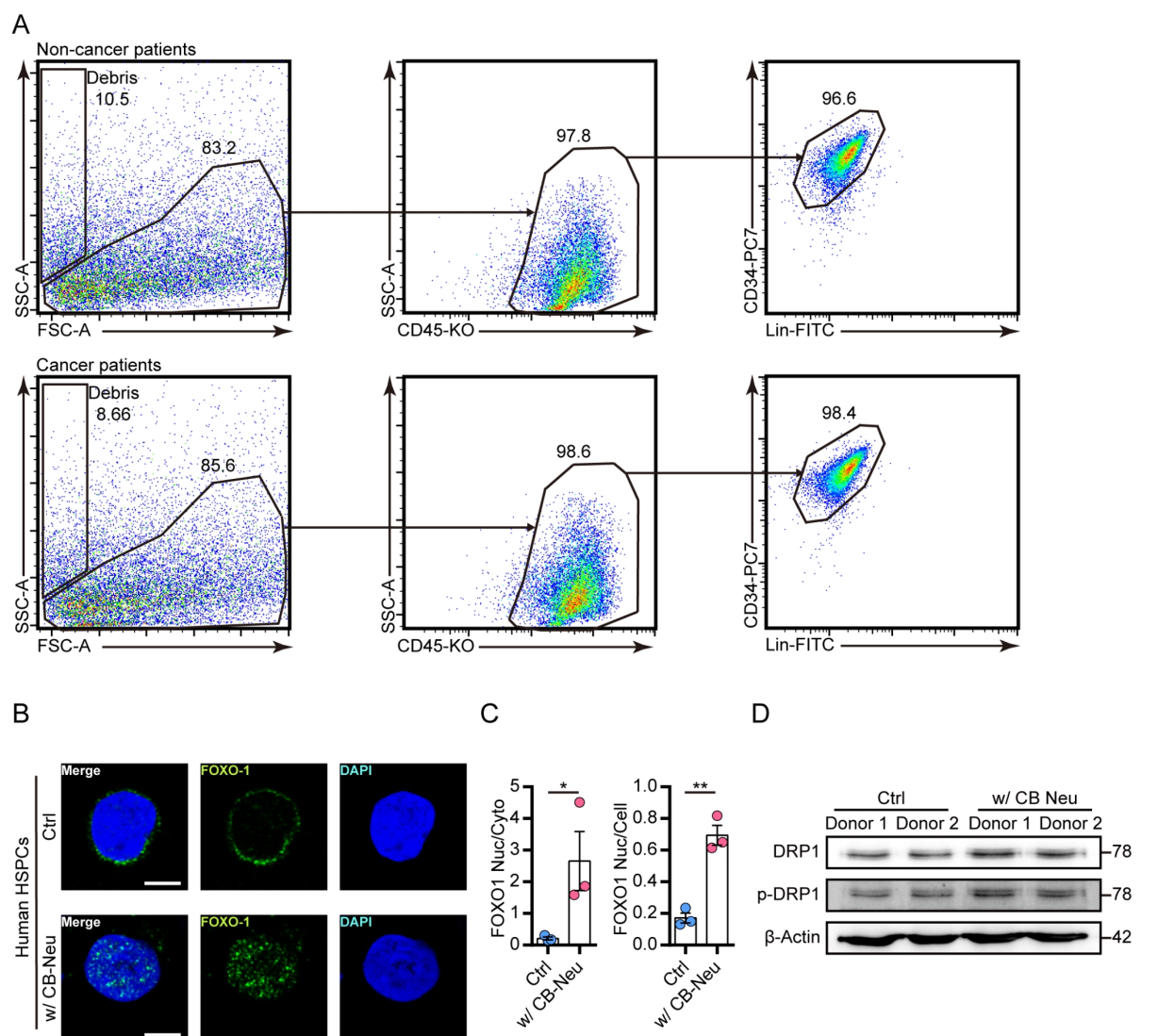
(B) BM LSK cells from healthy mice were cultured in vitro under conditions mimicking the splenic environments of tumor-free Ctrl or tumor-bearing Hepa mice (see Methods for splenic

stromal supernatant) with vehicle (Veh) or the DRP1 inhibitor Mdivi-1 (5 μ M). Percentage of GM-CSF⁺ LSK cells. **(C)** Representative flow cytometry plots showing the differential composition of Gr-1⁺ myeloid progeny generated under Ctrl and Hepa conditions. **(D)** Gr-1⁺ cells from the Hepa group were sorted into Ly6G⁺Ly6C^{low} neutrophil-like progeny and Ly6G⁻Ly6C^{high} monocytic progeny. Representative flow cytometry plots show the post-sort purity of each population, with numbers indicating the percentage of cells within the indicated gates. **(E)** Representative CFSE dilution profiles of CD8⁺ T cells co-cultured with sorted Ly6G⁺Ly6C^{low} neutrophil-like progeny (red, upper row) or Ly6G⁻Ly6C^{high} monocytic progeny (blue, lower row) from the Hepa group at the indicated myeloid cell-to-splenocyte ratios. Data are representative of $n = 3$ donors. **(F)** Immunoblotting of DRP1 in sg-Ctrl- and sg-*Dnm1l*-transduced LSK cells from Cas9-tdTomato knock-in mice. **(G)** Percentage of GM-CSF-expressing cells in sg-Ctrl and sg-*Dnm1l* LSK population cultured in vitro under conditions mimicking the splenic environments of tumor free Ctrl or tumor-bearing Hepa mice ($n = 3$ per group). **(H)** Confocal images of sg-Ctrl and sg-*Dnm1l* LSK cells cultured in vitro under conditions mimicking the splenic environments of tumor free Ctrl or tumor-bearing Hepa mice, stained with MitoTracker Green and Hoechst; scale bars, 5 μ m. Data are representative of $n = 3$ donors. **(I-J)** Immunoblotting of STING, p-STING, TBK1, p-TBK1, I κ B α , p-I κ B α , p65, p-p65 in BM and SP LSK cells from Hepa mice ($n = 3$ samples per group). Data are mean $\pm$ SEM. Statistics: two-way ANOVA corrected by Sidak's **(G)** post-hoc test. ** $P < 0.01$; *** $P < 0.001$.



Supplemental Figure 6. No significant changes in lymphocyte compartments following spleen- or tumor- targeted treatments and longitudinal tumor imaging.

(A-B) Number (A) and percentage (B) of splenic B cells and T cells in Hepa mice following pump-mediated delivery of vehicle (Veh), NAC, HLZ to the spleen ($n = 5-8$ mice per group). (C) Bioluminescence images of Hepa mice following pump-mediated delivery of Veh, NAC, or HLZ into the spleen. (D-E) Number (D) and percentage (E) of splenic B cells and T cells in Hepa mice following untreated or pump-mediated delivery of Mdivi-1 into the tumor (TU) or spleen (SP) ($n = 4$ mice per group). (F) Bioluminescence images of Hepa mice following untreated or pump-mediated delivery of Mdivi-1 to the tumor (TU) or spleen (SP). Data are mean $\pm$ SEM. Statistics: two-way ANOVA corrected by Sidak's (A), Dunnett's (B), or Tukey's (D, E) post-hoc test.



Supplemental Figure 7. Neutrophil-derived oxidative stress is associated with FOXO1 nuclear translocation and DRP1 activation in human CD34⁺ progenitors.

(A) Representative flow cytometry plots showing debris exclusion and the purity of CD45⁺Lin⁻CD34⁺ cells within MACS-enriched human splenic CD133⁺ cell preparations from the spleens of non-cancer control (upper) and cancer (lower) patients. Numbers denote the fraction of events within the indicated parent gate. FSC, Forward Scatter; SSC, side scatter; KO, Krome Orange; FITC, fluorescein isothiocyanate; PC7, phycoerythrin-cyanine 7. (B-C) Confocal images (B) and quantification (C) of FOXO1 localization in human CD34⁺ CB-HSPCs co-cultured with CB-derived CD15⁺ neutrophil-like cells ($n = 3$ per group); Scale bars, 5 μ m. (D) Immunoblotting of DRP1 and p-DRP1 in human CD34⁺ CB-HSPCs co-cultured with

CB-derived CD15⁺ neutrophil-like cells ($n = 3$ per group). Data are mean $\pm$ SEM. Statistics: paired Student's t test (C). * $P < 0.05$; ** $P < 0.01$.

Supplemental tables

Supplemental Table 1. Clinical information of the 6 patients providing spleen samples

Patient number	Age	Gender	Clinical diagnosis
# 1	70	Female	Liver cirrhosis; Hypersplenism
# 2	72	Male	Liver cirrhosis; Hypersplenism
# 3	50	Female	Splenic mass
# 4	49	Male	Hereditary spherocytosis; Splenomegaly
# 5	54	Male	Hepatocellular carcinoma; Splenomegaly
# 6	60	Male	Hepatocellular carcinoma; Hypersplenism

Supplemental Table 2. Fluorochrome-conjugated antibodies used for flow cytometry

Antigen	Fluorochrome	Clone	Cat. No.	Supplier	Reactivity
CD34	PE/Cyanine7	581	343515	Biolegend	Human
CD33	APC	WM53	303408	Biolegend	Human
CD14	Alexa Fluor 700	M5E2	557923	BD	Human
CD15	EF450	MMA	48-0158-42	eBioscience	Human
CD90	APC	5E10	559869	BD	Human
CD123	PE-CF594	7G3	562391	BD	Human
CD45	Krome Orange	J33	B36294	Beckman	Human
Lineage cocktail	FITC		348801	Biolegend	Human
CD45RA	Brilliant Violet 421	HI100	304130	Biolegend	Human
CD38	PE	HIT2	303506	Biolegend	Human
Arginase 1	PE	14D2C43	369704	Biolegend	Human
CD11b	PE/Cyanine7	ICRF44	982608	Biolegend	Human
CD15	Brilliant Violet 421	W6D3	323040	Biolegend	Human
CD115	APC	9-4D2-1E4	347306	Biolegend	Human
B220	Brilliant Violet 421	RA3-6B2	103240	Biolegend	Mouse
CD117	Brilliant Violet 421	2B8	105828	Biolegend	Mouse
GM-CSF	Brilliant Violet 421	MP1-22E9	564747	BD	Mouse
CD150	Brilliant Violet 421	TC15-12F12.2	115926	Biolegend	Mouse
Ly6C	Brilliant Violet 421	HK1.4	128032	Biolegend	Mouse
CD11b	PE/Cyanine7	M1/70	101216	Biolegend	Mouse
CD3	PE	17A2	100206	Biolegend	Mouse
CD135	PE-CF594	A2F10.1	562537	BD	Mouse
CD8a	eFluor 450	53-6.7	48-0081	eBioscience	Mouse
CD4	PE/Cyanine7	RM4-5	100422	Biolegend	Mouse
CD34	Alexa Fluor 647	RAM34	560230	BD	Mouse
CD45.2	Alexa Fluor 700	104	109822	Biolegend	Mouse
CD45	Brilliant Violet 570	30-F11	103136	Biolegend	Mouse
CD45.1	FITC	A20	110706	Biolegend	Mouse
CD117	PE/Cyanine7	2B8	558163	BD	Mouse
Sca-1	FITC	W18174A	160908	Biolegend	Mouse
Sca-1	AF700	D7	56-5981-82	ThermoFisher	Mouse
IFN- γ	APC	XMG1.2	505810	Biolegend	Mouse
Ly6C	APC/Cyanine7	HK1.4	128026	Biolegend	Mouse
MHC-II	Brilliant Violet 421	M5/114.15.2	107632	Biolegend	Mouse
NK1.1	Alexa Fluor 700	S17016D	156512	Biolegend	Mouse
Lineage cocktail	APC		558074	BD	Mouse
Lineage cocktail	PE/Cyanine 5.5		561301	BD	Mouse
Gr-1	APC	RB6-8C5	108412	Biolegend	Mouse
Ly6G	PE-CF594	1A8	562700	BD	Mouse
TNF- α	PE/Dazzle™ 594	MP6-XT22	506346	Biolegend	Mouse

Supplemental Table 3. Immunohistochemistry antibodies

Antigen	Clone	Cat. No.	Supplier	Host	Reactivity
CD117	Polyclonal	AF1356	R & D	Goat	Human/Mouse
CD31	D8V9E	#77699	Cell Signaling Technology	Rabbit	Mouse
Ly6G	1A8	127602	BioLegend	Rat	Mouse
4-HNE	HNEJ-2	ab48506	Abcam	Mouse	—

Supplemental References

1. Liu M, et al. PERK reprograms hematopoietic progenitor cells to direct tumor-promoting myelopoiesis in the spleen. *J Exp Med*. 2022;219(4):e20211498.
2. Wu C, et al. Spleen mediates a distinct hematopoietic progenitor response supporting tumor-promoting myelopoiesis. *J Clin Invest*. 2018;128(8):3425-3438.
3. Wu W-C, et al. Circulating hematopoietic stem and progenitor cells are myeloid-biased in cancer patients. *Proc Natl Acad Sci U S A*. 2014;111(11):4221-4226.