Supplementary Materials for

Aged neutrophils form mitochondria-dependent vital NETs to promote breast cancer lung metastasis

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Supplementary Materials and methods

1. Human samples

Peripheral blood (PB) was obtained from patients with breast cancer and breast fibroadenoma at the Second Affiliated Hospital of Zhejiang University School of Medicine from January 2017 to July 2020. None of the enrolled patients had previously received therapy, including surgery, radiotherapy or chemotherapy. Recruited patients were negative for hepatitis C virus, hepatitis B virus, HIV, and syphilis. Blood samples were collected in heparin-coated tubes (BD Vacutainer) and rapid serum tubes (BD Vacutainer) at 6:00 a.m. by licensed practical nurses. Pathology slides of primary breast cancer tissue, breast fibroadenoma tissue, lung tissue biopsy samples from breast cancer lung metastasis, normal lung tissue 6 cm away from primary lung cancer (tumor site <10 mm) from the radical resection sample were obtained. Detailed information of the characteristics of enrolled patients was provided in *online supplemental tables 2-4*. Informed consent forms were signed by all included patients and the procedure was approved by Ethics Review Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine and in compliance with the Declaration of Helsinki.

2. Cell culture

The 4T1 mouse mammary tumor cell line, MCF-7 and MDA-MB-231 human breast adenocarcinoma cell line were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Science (SIBS, Shanghai, China). The Py230 mouse mammary tumor cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were authenticated by STR profiling and confirmed negative for mycoplasma. Cells were cultured at 37°C in a 5% CO_2 cell culture incubator with RPMI-1640 medium containing 10% fetal bovine

serum (FBS, Gibco), 2 mM L-glutamine (Gibco) and 1% penicillin-streptomycin (Gibco).

A lentivirus system was used to construct shNAMPT-expressing, GFP-expressing, and luciferase-expressing stable cell lines. The NAMPT shRNA lentiviral plasmids were purchased from Genomeditech (Shanghai, China). GFP-expressing and GFP & luciferase-expressing lentiviruses were purchased from Zorin (Shanghai, China). For infection, the viral stock was supplemented with 6 μg/mL polybrene (Sigma-Aldrich), and successfully transfected cells were selected using puromycin (2 μg/mL, Beyotime, #ST551) for stable GFP-expressing and GFP& luciferase-expressing cells or blasticidin S (10 μg/mL, Genomeditech, #GM-040404) for stable shNAMPT-expressing cells. The *NAMPT shRNA* oligonucleotide sequences were as follows: *NAMPT shRNA1*: *5'-GATCCGCCACCTTATCTTAGAGTCATTCTCAAGAGGAATGACTCTAAGATAAGGTGGCTTTTTTG-3'*; *NAMPT shRNA2: 5'-GATCCGGCCACCTTATCTTAGAGTCATCTCAAGAGGATGACTCTAAGATAAGGTGGCCTTTTTTG-3'; and NAMPT shRNA3: 5'-GATCCGGCCAGATTATTTGTTGGTTTACTCAAGAGGTAAACCAACAAATAATCTGGCCTTTTTTG-3'*. A non-silencing *shRNA* (control *shRNA*) were used as a mock-transfected control (target sequence: *5'-TTCTCCGAACGTGTCACGTAA-3'*). Then, NAMPT expression was verified using real-time PCR and western blotting.

3. Mice

Wild-type BALB/c and BALB/c-nude female mice (6-8 weeks) were purchased from Slaccas (Shanghai, China). FVB/N-Tg (MMTV-PyMT) mice were purchased from Gempharmatech (Nanjing, China). All mice were housed in the specific pathogen-free animal room of Zhejiang Chinese Medical University at a constant temperature (22±2°C) and on a 12/12-h day/night cycle.

Genotyping of FVB/N-Tg (MMTV-PyMT) mice were performed according to the protocol provided by Jackson Laboratory. Briefly, mouse gDNA was extracted from mouse toe tissue using an Animal Genomic DNA Isolation Kit (Sangon Biotech, #B518221), and further amplified using PrimerSTAR Max DNA Polymerase (TaKaRa, #R045A) with specific primers. The amplified DNA was then detected using agarose gel electrophoresis (Bio-Rad) on a 1.5% agarose gel (Sigma-Aldrich, #A9539).

BALB/c mice were anesthetized with 0.8% sodium pentobarbital (*i.p.*, 80 mg/kg) and inoculated with a suspension of $1x10^5$ 4T1 cells in the right fourth mammary fat pad to establish the 4T1 tumor-bearing mouse model. All animal procedures were approved by the Ethics Review Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine.

4. Mouse irradiation and hematopoietic stem cells transplantation (HSCT)

In order to structure bown marrow SIRT1- knockdown mice, naive BALB/c recepient mice were suffered 7.0 Gy total body irradiation (3.5 Gy × 2 with a 24 h interval) and then gavaged with Sulfamethoxazole (50mg/kg) and Trimethoprim (10mg/kg) for 1 week in case of infection.

To obtain SIRT1- Knockdown hematopoietic stem cells (HSCs), naive BALB/c donor mice were sacrificed and the bone marrow cells from hind limbs was extracted. The cells were then stained with biotin anti-mouse CD117 (c-kit, Biolegend, #105803) Abs for 15 min and then washed with MACS buffer and stained with MojoSort™ Streptavidin Nanobeads (Biolegend, #480016) for 15 min. Magnetic beads separation was operated and the CD117 positive HSCs were isolated. The obtained cells were planted into 6-well plate with complete RPMI-1640 medium and shSIRT1 or shControl lentivirus (MOI=100) with polybrene (2μg/mL) was added. The plate was centrifuged at 200 g for 3 h and cultured in cell incubator for another 5h and fresh complete RPMI-1640 medium

was added. The cells were adjusted to $2x10^6$ / 200μL and injected into recepient mice 4h after last irradiation (*i.v.*). The irradiated recepient mice were given sulfamethoxazole for 1 more week and then were inoculated with a suspension of $1x10^5$ 4T1 cells in the right fourth mammary fat pad to establish the 4T1 tumor-bearing mouse model.

The SIRT1 shRNA lentiviral plasmids were purchased from Zorin (Shanghai, China). The SIRT1 shRNA oligonucleotide sequences were as follows: SIRT1 shRNA1: 5'-GATCCGAGTGAGACCAGTAGCACTAATCTCAAGAGGATTAGTGCTACTGGTCTCACTCTTTTTTG-3'; SIRT1 shRNA2: 5'-GATCCGGCCATGTTTGATATTGAGTATCTCAAGAGGATACTCAATATCAAACATGGCCTTTTTTTG-3'; and SIRT1 shRNA3: 5'-GATCCGGAGGGTAATCAATACCTGTTTCTCAAGAGGAAACAGGTATTGATTACCCTCCTTTTTTTG-3'. A non-silencing shRNA (control shRNA) were used as a mock-transfected control (target sequence: 5'-TTCTCCGAACGTGTCACGTAA-3').

5. Neutrophils depletion *in vivo*

The experiment described below was conducted to evaluate the effect of a neutrophil deficiency on the lung metastasis of breast cancer. The anti-mouse Ly-6G antibody (BioXcell, #BP0075-1) was administered at a dose of 1.5 mg/kg/every other day (*i.v.*); PBS was administered as a control. The treatment time is shown in *online supplemental figure 1D*. No neutrophils were detected in lung tissues at 24 h after the injection using flow cytometry (*online supplemental figure 1E*). At the end of the treatment, the mouse lung tissues were fixed with 10% formalin for H&E staining.

6. Electron microscopy

For scanning electron microscopy (SEM) of sorted neutrophils, sorted neutrophils subsets were

seeded on poly-D-lysine (PDL, Beyotime, #ST508) pre-coated sterile round glasses placed in a 12-well plate in complete RPMI-1640 medium and allowed to adhere for 1-3 h (depending on the adhesion degree observed under an optical microscope). For SEM of tissues, formalin-fixed and paraffin-embedded tissues were cut into 3-5-μm-thick continual sections (immunohistofluorescence staining was applied for cell localization). Then, the tissues on the glasses were covered with 2.5% glutaraldehyde and incubated for > 4 h, followed by an incubation with 1% tannic acid for 2 h. Subsequently, samples were gradually dehydrated in ethanol solutions and isoamyl acetate 2 times for 15 min each. Finally, critical point drying and ion sputtering gold coating were performed. Images were captured by Nova NanoSEM 450 (FEI, Thermo Fisher, USA) and processed by xT microscope server software.

For transmission electron microscopy (TEM), sorted neutrophil subsets were centrifuged and the cell pellet was suspended in 2.5% glutaraldehyde and incubated for > 4 h and incubated with 1% tannic acid for 2 h in a 1.5-mL tube. Then, cells were dehydrated as described above. Subsequently, cells were sequentially embedded in embedding agent plus acetone (2:1 at 37°C for 1 h, 1:1 at 37°C for 1 h) and pure embedding agent at 37°C for 1 h followed by an incubation at 37°C overnight. Ultrathin slices were generated and positive staining was performed. Images were captured by Tecnai 10 (Thermo Fisher, USA).

7. Giemsa staining

Collected neutrophils were initially counted and adjusted to a density of $1x10^6$ cells/mL. 50 μL of the cell suspension was added to a cytospin apparatus and cells were attached to the slide. Then, cells were fixed with 4% paraformaldehyde (PFA) for 20 min, stained with Giemsa solution (Solarbio, #G1010) for 10 min, and then washed with flowing ddH_2O (pH=6.8-7.2). Cells were air-dried and observed under an optical microscope. Nucleus morphology was analyzed and counted in

randomly selected fields of view.

8. RNA isolation and quantitative real-time PCR

Mouse tissues were ground in liquid nitrogen firstly and cell samples were added directly to TRIzol reagent (Invitrogen, #15596-018). Total RNA was extracted according to the manufacturer's instructions. The concentration and purity of all RNA samples were determined using a NanoDrop spectrophotometer (Thermo Fisher, USA). The cDNA templates were synthesized using PrimeScript RT Master Mix (TaKaRa, #RR036A). Quantitative PCR was performed with gene-specific primers and TB Green Premix Ex Taq (TaKaRa, #RR420A) using a 7500 Fast Real-Time system (Applied Biosystems, USA). Data were processed using 7500 (V2.3) software (Applied Biosystems). Results were normalized to the housekeeping gene *β-actin* and then reported as fold up-regulation compared with the control.

9. Sample preparation and RNA sequencing

For the acquisition of inflammatory aged neutrophils, LPS (2.5 mg/100 μL, Sigma-Aldrich, #L2880) was administrated through the trachea of 8-week-old female BALB/c mice and lung tissue was obtained 12 h later. For the acquisition of tumor-associated aged and non-aged neutrophils, lung tissue was obtained from female BALB/c mice at 2 weeks after the tumor cell inoculation. A single cell suspension from the lung tissue was generated and cell surface marker staining was performed as described above. FACS sorting was conducted to obtain the target cell subpopulation. The following FACS sorting strategy was used: CD45-APC/Cy7, CD11b-PE/Cy7, Ly6G-FITC, CXCR4-APC, and CD62L-PE.

Total RNA was extracted from sorted neutrophil subsets using TRIzol reagent. The mRNAs with a polyA tail were enriched using magnetic beads coupled with OligodT, rRNA was removed through

hybridization with a DNA probe and selective digestion of the DNA/RNA hybrid strand by RNaseH, and then the DNA probe was digested with DNaseI. The obtained RNA was fragmented using interrupting buffer, and the random N6 primer was used for reverse transcription to synthesize the cDNA double strand to form double-stranded DNA. The synthetic double-stranded DNA ends were flattened and phosphorylated at the 5' end, and the 3' end formed a sticky end with a protruding "A"; then, a bubbling joint with a convex "T" at the 3' end was attached. The PCR ligation product was amplified with specific primers and heat-denatured into a single strand, and the single-stranded DNA was cyclized with a bridge primer to obtain a single-stranded circular DNA library. The BGISEQ-500 platform (Beijing Genomics Institute, BGI) was applied for sequencing. The result was initially subjected to quality control and aligned to the reference genome. The second quality control step was performed and then a quantitative analysis of gene expression levels (principal components, correlation, differential gene screening, etc.) and differentially expressed genes between selected samples, along with the significant functional enrichment analysis of GO functions and KEGG pathways were performed.

10. Separation of high-density and low-density neutrophils

Lung tissue was obtained from 4T1-tumor bearing mice 2 weeks after the tumor cells were inoculated and digested into a single cell suspension as described above. The procedure for separating LDNs and HDNs was described in a previous study [40]. Briefly, 3 mL of Histopaque-1119 (Sigma-Aldrich, #11191) were pipetted in a sterile 15-mL tube and carefully overlaid with 3 mL of Histopaque-1077 (Sigma-Aldrich, #10771); then, 6 mL of the sample-BSA mixture were slowly overlaid on the Histopaque, followed by centrifuge at 700 g for 30 min at RT with no brake. Two obvious cell rings were observed at different liquid levels and aspirated. The aspirated cell suspension was further purified with magnetic isolation to obtained neutrophils. Neutrophils in the

upper cell suspension were regarded as LDNs and neutrophils in the lower cell suspension were regarded as HDNs.

11. *In vitro* co-culture of neutrophil subsets with T cells or tumor cells

T cells were isolated from the spleen of naïve BALB/c mice using the mouse CD3 selection kit according to the manufacturer's instructions (Biolegend, #480100). Aged and non-aged neutrophils were obtained via FACS from the lungs of 4T1 tumor-bearing mice at 2 weeks after inoculation. LDNs and HDNs were obtained as described above. BM-derived neutrophils (defined as PMN-MDSCs) were obtained via MACS from the BM of 4T1 tumor-bearing mice 2 weeks after inoculation. For the analysis of intracellular IL-2 and IFN-γ secretion from T cells, T cell and neutrophil subsets (cell ratio of 1:10) suspended in complete RPMI-1640 medium were plated into U-bottom 96-well plates with the Ultra-LEAF™ purified anti-mouse CD3ε antibody (Biolegend, Clone 145-2C11, #100340) and Ultra-LEAF™ purified anti-mouse CD28 antibody (Biolegend, Clone 37.51, #102116). Then, cells were harvested on day 3. The cell stimulation cocktail plus protein transport inhibitors (eBioscience, #00-4975-03) were added and incubated for 5 h; The intracellular staining procedure was described above. For the T cell proliferation assay, isolated T cells were labeled with CFSE (1 μM, Biolegend, #423801) at 37°C for 10 min and thoroughly washed 3 times with pre-warmed complete RPMI-1640 medium. Then, CFSE-labeled T cells and neutrophils (cell ratio of 1:10) were plated into U-bottom 96-well plates and incubated with Ultra-LEAF™ purified anti-mouse CD3ε, anti-mouse CD28 antibodies and recombinant murine IL-2 (Peprotech, #212-12). Cells were harvested at day 3 and examined using flow cytometry.

CCK8 and CFSE assays were performed separately to assess the effects of neutrophil subsets on the proliferation of tumor cells. For the CCK8 assay (Dojindo Laboratories, #CK04), 4T1 tumor cells were equally separated into three groups (1. control, 2. co-cultured with aged neutrophils, and 3.

co-cultured with non-aged neutrophils) at different cell ratios. Neutrophils were added until 4T1 cell attachment was observed. The medium was changed to remove cultured neutrophils after co-cultured for 48 h, CCK8 solutions with fresh complete RPMI-1640 medium were added to each well and incubated for 2 h at 37°C. Then, absorbance was measured at 450nm by SpectraMax M5 (Molecular Devices, USA). For the CFSE assay, 4T1 tumor cells were labeled with CFSE and then equally separated as described above. Cells were harvested on days 3 and 5 and analyzed using flow cytometry.

12. Biochemical characterization and detection of neutrophil subsets

Arginase1, Prostaglandin E2 (PGE2), nitric oxide (NO), reactive oxygen species (ROS), reactive nitrogen species (RNS) levels were measured to evaluate the cellular immunosuppressive function. Arginase Activity Assay Kit (Sigma-Aldrich, #MAK112), Prostaglandin E2 Assay (R&D Systems, #KGE004B), Total Nitric Oxide Assay Kit (Beyotime, #S0023), Reactive Oxygen Species Assay Kit (Beyotime, #S0033) and Reactive Nitrogen Species Detection Kit (Bestbio, #BB-470567) were applied. The procedures were performed according to the manufacturers' instructions.

13. Induction of neutrophils *in vitro*

Primary mice neutrophils or human neutrophils were induced with SRT1720 (0.01- 10 μM and 1 μM if not specified otherwise, Selleck, #S1129), Mdivi-1 (10 μM, Sigma-Aldrich, #M0199), PMA (20 nM, Abcam, #ab120297), mPTP inhibitors such as TRO19622 (1 μM, MCE, #HY-14796) and cyclosporin A (100 nM, Cell Signaling Technology, #9973s), ionomycin (10 μM, Abcam, #ab120370), recombinant murine GM-CSF (0.5 μg/mL, Peprotech, #315-03), recombinant murine and human NAMPT (0.5 μg/mL, Biovision, #4908-10 and #4907-10), EX527 (10 μM, Selleck, #S1541), FK866 (10 nM, Selleck, #S2799), Apocynin (10 μM, Selleck, #S2425), or DNaseI (100 U/mL, Roche,

#11284932001) for 4 h and harvested for flow cytometry, western blotting and real-time PCR.

14. Neutrophil electroporation

The procedure used for cell electroporation was performed with the HiCEP system. Briefly, primary BM-derived neutrophils were resuspended in 500 μL of electroporation buffer, and then the appropriate amount of the siControl or siLBR plasmid was added (final concentration of the plasmid was 20 μg/mL; final concentration of the siRNA was 100 nM). After mixing thoroughly by pipetting up and down several times, the cell suspension was transferred to an electrode cup (Bio-Rad). The parameters were set (voltage: 300 V, capacitance: 500 μF, pulse duration: 5 ms, cell density: $1x10^7$/mL) and electroporation was conducted. After the electric shock was administered, the cup was placed in a constant temperature incubator for 8-12 min to allow the nucleic acid to fully enter the cell. Then, the cell suspension was inoculated in preheated medium containing recombinant GM-CSF (0.5 μg/mL) for 24 h and cells were harvested for protein extraction and western blotting. For the SRT1720 induction assay, SRT1720 was added to culture medium and cells were cultured for another 4 hours. The *LBR* siRNA oligonucleotide sequence was *5'-CCGGCCTGCCATACTTCTACATTATCTCGAGATAATGTAGAAGTATGGCAGGTTTTTG-3'*.

15. Western blot

Total proteins were harvested from sorted neutrophils, cultured tumor cells, or fresh frozen tissues and lysed with pre-cooled lysis buffer supplemented with a cocktail of protease and phosphatase inhibitors (Thermo Fisher, #78445). Protein concentrations were measured using a bicinchoninic acid (BCA) assay kit (Thermo Fisher, #23227). The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After blocking with 5% (w/v) fat-free milk (BD Biosciences,

#232100) at RT for 1 h, the membrane was incubated with the corresponding primary antibodies overnight at 4°C followed by an incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactive bands were identified using enhanced chemiluminescence (Thermo Fisher, #32109). Primary Abs specific for SIRT1 (1:2000, Abcam, #ab110304), LBR (1:2000, Abcam, #ab122919), C/EBPε (1:100, Santa, #sc-515192), LC-3B (1:2000, Abcam, #ab192890), NAMPT (1:1000, Abcam, #ab236874), Cit-Histone H3 (1:1000, Abcam, #ab5103), β-Actin (1:2000, HuaBio, #EM21002), GAPDH (1:2000, HuaBio, #ET1601-4) and β-Tubulin (1:5000, HuaBio, #EM0103) were used. Secondary Abs including goat anti-rabbit IgG-HRP (1:10000, HuaBio, #HA1001) and goat anti-mouse IgG-HRP (1:10000, HuaBio, #HA1006) were applied. Software ImageJ (version 1.48) was used for quantification of WB images.

16. Co-immunoprecipitation (Co-IP)

Cell samples were harvested, then washed twice with cold PBS and lysed in lysis buffer at 4°C for 30 min. Cell lysates were centrifuged at 14000 g for 15 min. The supernatant was divided into two parts: 0.8 mL for IP and 0.1 mL for the input. IP cell lysates were incubated overnight with primary antibodies and further incubated with Protein A/G Magnetic Beads (Thermo Fisher, #88802) for 4 h at 4°C to capture the antigen-antibody complex. The magnetic bead-antigen-antibody complex was harvested by capture with a magnetic rack (Invitrogen) and washed 3 times with cold PBS. The bound proteins were boiled in SDS sample buffer and resolved on 10% SDS-PAGE gels for western blotting. Primary Abs specific for SIRT1 (1:2000, Abcam, #ab110304), C/EBPε (1:100, Santa, #sc-515192), β-actin (1:2000, HuaBio, #EM21002), and acetyl Lysine (1:1000, Abcam, #ab21623) were used.

17. Chromatin immunoprecipitation (ChIP)

The ChIP procedure was performed according to the manufacturer's protocol (ChIP kit, BersinBio, #Bes5001). Treated neutrophils were harvested and washed with PBS twice. Next, $1x10^7$ cells were resuspended and crosslinked in 10 mL of PBS containing 1% formaldehyde for 10 min at RT. Then, 1 mL of glycine (1.375 M) was added to neutralize the cross-links for 5 min on ice and then washed with pre-cooled PBS twice. For cell lysis, cells were resuspended in lysis buffer containing protease inhibitors and DTT and lysed by sonication. The lysed sample was then incubated with agarose beads for 60 min at 4°C. The supernatant was divided into two parts: 0.8 mL for IP and 0.1 mL for the input control. Elution buffer was added to the input sample and stored at -20°C. The IP sample was incubated with an antibody specific for C/EBPε (1:100, Santa, #sc-515192) at 4°C overnight. IgG (1:1000, Abcam, #ab171870) and an antibody against Histone H3 (1:500, Abcam, #ab1791) were used as negative and positive controls respectively. Then, Protein A/G Magnetic Beads (Thermo Fisher, #26162) were added and incubated for 30 min at RT. Beads were collected using a magnetic rack followed by washes with TE wash buffer and elution with elution buffer. Cross-links were reversed at 65°C for 6h and DNA was subsequently purified. DNA was amplified and visualized using gel electrophoresis. The sequences of the primers used were: mouse *LBR* promoter region forward: *CTCGGAGTAGGATTCGTCTTTAAG*, mouse *LBR* promoter region reverse: *GATTTGTCATTGCCGTTGGG*, mouse *LBR* promoter region upstream forward: *AGATTTCCACCCAGGGCAAC*, mouse *LBR* promoter region upstream reverse: *AGTGAGTAAGTGCGTGCCTC*, mouse *LBR* promoter region downstream forward: *CCTCCGGAGTGTGTCTTTGT*, and mouse *LBR* promoter region downstream reverse: *AAGCCAGTGCTCTGATACCAA*.

18. NADP/NADPH assay

The NADP/NADPH assay was performed in various neutrophil subsets according to the protocol

provided with the NADP/NADPH assay kit (Abcam, #ab65349). Briefly, cells were harvested, washed with cold PBS, and then extracted with NADP/NADPH Extraction Buffer by performing two freeze/thaw cycles (20 min on dry ice followed by 10 min at RT). The supernatant was obtained and divided into 2 samples: 1. a total NADP & NADPH sample and 2. a NADP decomposed sample. The reaction mix was added and incubated at RT for 5 min. NADPH Developer was then added and incubated for 1-4 h. Multiple readings of the plate were recorded for 1-4 h at an OD of 450 nm using a microplate reader.

19. Detection of mitophagy in neutrophils

Mitophagy was detected in neutrophils using the protocol provided by Mitophagy Detection Kit (Dojindo, #MD01). The mitophagy working solution was added to the cell suspension at a final concentration of 0.1 μM, followed by an incubation at 37°C for 30 min. Cells were then used for *in vitro* stimulation after two washes with an HBSS solution. Pepstatin A (10 μM, Sigma-Aldrich, #P5318) and leupeptin (100 μM, Sigma-Aldrich, #L2884) were used to inhibit mitophagosome clearance. Samples were observed using a fluorescence microscope or detected using flow cytometry (by PE/Cy 5.5).

20. Neutrophils lifespan evaluation

Aged and Non-aged neutrophils were obtained from the lungs of 4T1 tumor-bearing mice at 2 weeks after inoculation via FACS sorting and plated into 96-well plate with complete RPMI-1640 medium. Neutrophil lifespan was evaluated by two methods. (1) Cells were harvested and re-suspended in detection buffer and incubated with Annexin V-FITC and 7-AAD (Biolegend, #640922) in the dark for 15 min at RT. For the flow cytometry analysis, samples were re-suspended in 300 μL of cell staining buffer. (2) cells suspension (200 μL) was harvested and mixed with 10 μL

CountBright™ Plus Absolute Counting Beads ($1x10^4$ beads/10 µL, Thermo Fisher, #C36995) and detected using flow cytometry. Absolute count of viable neutrophils (cells/ µL) was calculated by cell count/ counting beads count and multiplied by $1x10^4$ beads/10 µL.

21. Immunocytofluorescence (ICF)

Sorted neutrophil subsets were seeded on PDL-coated sterile round glasses in 12-well plates in complete RPMI-1640 medium and incubated for 1-3 h until cells adhesion. The cells on the glass were then fixed with 4% PFA for 10 min at RT, permeabilized with 0.2% TritonX-100 (Sigma-Aldrich, #X100) for 20 min, blocked with 3% BSA (MP Biomedicals, #0218054990) for 60 min, and incubated with the primary antibody at 4°C overnight. The fluorescent dye-conjugated secondary antibody was then added to the cells and incubated at 4°C for 2 h. After the washing step, DAPI (Invitrogen, #D1306) was added, and cells were mounted on a slide. The samples were analyzed with an LSM 710 confocal microscope (Carl Zeiss, Germany). Primary Abs specific for Cit-Histone H3 (1:200, Abcam, #ab5103), LBR (1:500, Abcam, #ab122919), α-tubulin (1:200, Abcam, #ab195887) and the fluorescent probes Mito-tracker Green (100 nM, Beyotime, #C1048) and Sytox Orange (1 µM, Thermo Fisher, #S11368) were applied. Secondary Abs specific for rabbit IgG labeled with Alexa Fluor® 488 (1:500, Abcam, #ab150077) and Alexa Fluor® 594 (1:500, Abcam, #ab150080) were applied. Software ImageJ (version 1.48) was used for quantification of images intensity.

22. Immunohistochemistry (IHC), immunohistofluorescence (IHF) staining and multiplex immunohistochemistry

IHC and IHF were performed using a standard protocol (Absin, #abs957). Briefly, 4-5 µm paraffin sections were deparaffinized through alcohol gradients and rehydrated with water. Antigenic

retrieval was performed using Tris-EDTA (pH=9) buffer in thermostatted bath (98°C) for 30 min. Primary Abs specific for NAMPT (1:2000, Abcam, #ab236874) and secondary Abs specific for rabbit IgG (HRP, 1:5000, HuaBio, #HA1001) and rabbit IgG (Alexa Fluor® 488, 1:500, Abcam, #ab150077) were applied. Slides were then scanned using a Pannoramic MIDI instrument (3DHISTECH Ltd.) and images were captured using Pannoramic Viewer software (3DHISTECH Ltd.). The IHC staining score was semi-quantitatively evaluated by determining the staining intensity and the percentage of positive cells. The two scores were added together to yield the immunoreactive score (IRS). Cases with discrepancies in the IRS were discussed with other pathologists until a consensus was reached. IHC staining was evaluated by two researchers (C. Yang and Z. Wang) who were blinded to the clinicopathological characteristics.

Multiplex IHC was performed using sequential staining cycles as described below. Briefly, formalin-fixed, paraffin-embedded tissue sections were first deparaffinized and underwent antigen retrieval. Sections were then blocked and stained with commercially available anti-GFP (1:1000, Abcam, #ab13970), anti-Neutrophil Elastase (1:100, Abcam, #ab68672), anti-CD62L (1:100, Abcam, #ab119834), anti-Pan Cytokeratin (1:500, Abcam, #ab215838), anti-MPO (1:50, Abcam, #ab9535) and anti-Ly-6G (1:100, Servicebio, #GB11229) antibodies at RT for 1 h. Next, sections were incubated with Opal Polymer HRP Ms + Rb (PerkinElmer, #ARH1001EA) at RT for 30 min. Tyramide signal amplification (TSA)-based visualization was performed with Opal fluorophores. A microwave treatment was performed to remove the antibody-TSA complex after every staining cycle and the process was completed with DAPI counterstaining.

23. Analysis of the ability of neutrophils to capture tumor cells

Aged and non-aged neutrophils were obtained from the lungs of 4T1 tumor-bearing mice at two weeks after inoculation via FACS sorting. Then, sorted cells were labeled with Sytox Orange,

adjusted to a density of $1x10^6$ cells/mL, seeded on PDL-coated glasses, and incubated for 3 h. GFP-expressing tumor cells ($1x10^6$/mL) were added to the wells via a micropump (5 mL/h). The process by which neutrophils captured tumor cells was recorded with a fluorescence microscope (Zeiss) through real-time video. Finally, the wells were gently washed with PBS and cells were fixed with 4% PFA for immunofluorescence staining or 2.5% glutaraldehyde for electron microscopy.

24. Cell-free DNA extraction and electrophoresis

Cell-free DNA (cfDNA) was extracted from the cell culture supernatant using the QIAamp Circulating Nucleic Acid Kit (Qiagen, #55114) and DNA concentrations were detected using the Picogreen dsDNA Quantitation Reagent (Yeasen, #12641ES01). DNA was amplified with the iTaq Universal SYBR Green mix (BioRad, #1725121) and specific primers including three human mitochondrial genes (*Cyb, Atp6, Co1*), three human nuclear genes (*Actb, Gapdh, Fas*), two mice mitochondrial genes (*16s, Cyto C*) and two mice nuclear genes (*18s, β2 microgolbulin*), and then detected using agarose gel electrophoresis on a 1% agarose gel.

25. Mitochondrial permeability transition pore (mPTP) assay

The opening of the mPTP in neutrophils was assessed using the Image-IT™ LIVE Mitochondrial Transition Pore Assay Kit (Thermo Fisher, #I35103). Briefly, neutrophils were harvested and washed with modified HBSS buffer twice before being incubated with a labeling solution (modified HBSS buffer) containing a 1.0 μM calcein AM stock solution, 0.2 μM MitoTracker Red CMXRos stock solution and 1.0 mM $CoCl_2$ for 15 min at 37˚C in the dark. Further wash steps were performed with modified HBSS buffer and cells were divided into different groups, including a control group, SRT1720 group and ionomycin group (positive control). A microplate reader (SpectraMax M5) was applied for the real-time detection (30-s intervals) using a 488 nm laser.

26. Cell adoptive transfer *in vivo*

The experiments described below were performed to evaluate the effects of aged and non-aged neutrophils on the formation of the pre-metastatic niche in the lung (online supplemental figure 5D). (1) Recombinant murine G-CSF (10 mg/kg, Peprotech, #250-05) was injected into 3-day tumor-bearing BALB/c female mice and PB was obtained after 3 h; (2) PB was obtained from 2-week 4T1 tumor-bearing mice. MACS isolation of neutrophils was performed on both samples. The former cells are regarded as non-aged neutrophils and the latter cells are regarded as aged neutrophils (confirmed by flow cytometry). Isolated neutrophils were adjusted to a density of $1x10^7$ cells/50 μL. Cells were injected into 4T1 tumor-bearing mice (female BALB/c or BALB/c nude mice) every other day (*i.v.*) for 2 weeks and 4T1 cells expressing luciferase ($1x10^6$/100 μL) were injected through the tail vein on day 14. *In vivo* imaging was performed after mice were injected D-Luciferin (*i.p.,* Promega, #E1601) at the indicated time point (2 h) to observe the signal intensity of fluorescein using an IVIS Lumina LT apparatus (PerkinElmer, USA).

Next, 4T1 cells expressing luciferase ($1x10^6$/100 μL) were injected through the tail vein of naïve and 2-week 4T1 tumor-bearing mice and *in vivo* imaging was performed again at the indicated time point (2 h) to observe the signal intensity of fluorescein and to evaluate the difference in tumor metastasis between naïve and tumor-bearing mice.

27. Measurement of extracellular NAMPT (eNAMPT) levels

For the eNAMPT measurement, $5x10^5$ cells were seeded in 6-well plates and incubated for 24 h. Cells were incubated with serum-free medium for 24 h, and the resulting conditioned medium was collected. The conditioned medium was then concentrated by centrifugal filter units (Amicon Ultra-15, 3 KDa, Merck Millipore, #UFC900396) until 50 μL remained and then analyzed using

western blotting. In parallel, samples were analyzed for eNAMPT concentrations using an ELISA kit based on the competitive enzyme immunoassay principle (Raybiotech, #EIA-VIS).

28. *In vivo* therapeutic intervention

For the flow cytometry analysis, the intervention was started after 4T1 tumor cells were inoculated into the mammary fat pad. FK866 (25 mg/kg, Selleck, #S2799), Mdivi-1 (50 mg/kg, Selleck, #S7162), TRO19622 (30 mg/kg, MCE, HY-14796) or DNAseI (2.5 mg/kg, Roche, #11284932001) was injected (*i.p.*) and Vitamin B3 (500 mg/kg, Beyotime, #S1761) was administered by gavage every day for 2 weeks. After 2 weeks, lung tissues and PB were obtained for subsequent flow cytometry analysis.

For *in vivo* imaging, luciferase-expressing 4T1 cells ($5x10^5$ cells/100μL) were injected (*i.v.*) 2 weeks after 4T1 tumor cells were inoculated in the mammary fat pad ($1x10^5$ cells/100μL), and then interventions were started. The intervention strategy is described above. After 2 weeks, mice were injected D-Luciferin and subjected to inhalation anesthesia with isoflurane followed by *in vivo* bioluminescence imaging using the IVIS Lumina LT instrument.

29. Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software 6.0, GraphPad) and SPSS (IBM SPSS version 22 for Windows, SPSS, Inc.). Data are presented as the mean ± standard deviation (SD). Paired and Unpaired two-tailed Student's t-test and Mann-Whitney U-tests were used for two-group comparisons. One-way analysis of variance (ANOVA) or two-way ANOVA followed by the Bonferroni's test was used for the multiple comparisons. Repeated-Measures ANOVA was used for changes over time in the groups. Differences were considered significant when $P<0.05$ and are indicated as ns, not significant, $*P<0.05$, $**P<0.01$,

***P<0.001. All *in vitro* experiments were performed at least three times with successful replication. Three or more biological replicates were used for every study and specific n values are listed in the figure legends.

Supplementary Table Legends

Table S1. Comparison of the biochemical and molecular parameters associated with MDSC characteristics and functions in lung LDNs, HDNs, aged neutrophils and non-aged neutrophils.

Table S2. Clinico-pathological characteristics of patients included in this study (Fig. 1I, 1J and 7H).

Table S3. Clinico-pathological characteristics of patients included in this study (Fig. 7G and S7I).

Table S4. Clinico-pathological characteristics of patients included in this study (Fig. 2D, 2E).

Supplementary Video Legends

Video S1: Real-time video of breast cancer patient PB-derived aged neutrophils capturing MDA-MB-231 cells. Green: GFP-expressing MDA-MB-231 cells, red: Sytox Orange-labeled neutrophils.

Video S2: Real-time video of lung aged neutrophils from 2-week tumor bearing moue capturing 4T1 cells. Green: GFP-expressing 4T1 cells, red: Sytox Orange-labeled neutrophils.

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