Supplemental Materials

Chemicals

Ponatinib was obtained from LC laboratories (Cat. #P-7022) and suspended in DMSO to prepare 10 mM stock for cell culture experiments. For mice treatment, ponatinib (15 mg/kg) was dissolved in a citrate buffer solution (pH 2.75). Dexamethasone was purchased form MedChemExpress (Cat. #HY-14648A) and dissolved in 10% DMSO + 40% PEG300 + 5% Tween-80 + 45% saline. Mice were given dexamethasone (2 mg/kg) via intraperitoneal injections three times a week. The direct NLRP3 inhibitor CY-09 was purchased from MedChemExpress (Cat. # HY-103666) and clear solution was prepared in 10% DMSO + 40% PEG300 + 5% Tween-80 + 45% saline. Mice were injected with CY-09 (2 mg/kg) six times a week via intraperitoneal injections. Paquinimod, a specific inhibitor of S100A8/S100A9 was obtained from MedChemExpress (Cat. # HY-100442) and 10mM stock solutions were prepared for *in vitro* experiments. For *in vivo* experiments, mice were administered with paquinimod in drinking water (3.75 mg/kg) ad libitum, as previously reported.[1, 2] TLR-4 inhibitor TLR-IN-C34 was purchased from Sigma-Aldrich (Cat. # SML0832), and 1 mg/ml stock solution was prepared for *in vitro* experiment. Lipopolysaccharides (LPS) was purchased from Sigma-Aldrich (Cat. # L4391), and a 5mg/ml stock solution was prepared for *in vitro* experiment.

Animals

Wild-type (WT) C57Bl/6J, ApoE null ($ApoE^{-/-}$), and $NLRP3^{-/-}$ mice (NLRP3tm1Bhk, 0213020) were purchased from Jackson Laboratories (Bar Harbor, ME). Both male and female mice were randomly allocated to experimental groups. That said, a similar distribution of males and females between the experimental groups was always ensured.

ApoE$^{-/-}$ mice were fed a HFD (42% from Fat, Teklad TD.88137, Envigo) for 8 weeks prior to receiving ponatinib and continued with HFD feeding during the course of treatment. ApoE$^{-/-}$ mice on HFD were 15 to 20 weeks of age, which is expected to represent early atherosclerotic development. Both age and sex-matched control groups were employed in all the animal studies. For *in vivo* dosing, ponatinib (15 mg/kg) was administered daily by oral gavage. Fresh stock solution was prepared every 7 d and stored at 4°C. The dose of ponatinib was based on prior studies showing therapeutic responses in preclinical cancer models during drug development.[3, 4] All mice were maintained in a pathogen-free environment at the University of Alabama (UAB) animal facilities. All animals were included in the analysis. All procedures were performed after approval from the Institutional Animal Care and Use Committees (IACUC), in agreement with the NIH Guide for the Care and Use of Laboratory Animals.

Cell Isolation and Culture

Neonatal rat ventricular cardiomyocytes (NRVMs) were isolated and cultured as previously described.[5] For all in vitro experiments with NRVMs, ponatinib dissolved in DMSO was diluted to the desired concentration in the appropriate cell culture media. For all controls, DMSO was added in an equivalent volume to the highest dose of ponatinib. Isolated mouse splenocytes, bone marrow (BM) cells, and human PBMC (iXCells Biotechnology, USA) were cultured in RPMI 1640 (Gibco, Invitrogen, UK) with supplementation of 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 µg/ml penicillin-streptomycin, and 0.1 mM non-essential amino acids (Life Technologies, Waltham, MA). K562 cells (ATCC #CCL243) were cultured in ATCC-

formulated Iscove's Modified Dulbecco's Medium (Catalog No. 30-2005) with supplementation of 10% fetal bovine serum (FBS).

For leukocyte isolation, mice hearts were perfused with 1X PBS, harvested, and minced. Minced hearts were suspended in digestion media prepared in RPMI 1640 containing collagenase I (C0130, 1mg/ml), collagenase XI (C7657, 0.1mg/ml), hyaluronidase (H3506, 0.1mg/ml), and DNase I (D4527, 1ul/ml), digested the tissue in a water bath (37ºC) for 1 hour with gentle shaking. After 1 hour of tissue digestion, the digested contents were filtered with 70µm strainers. 5% FBS containing RPMI 1640 media were added to neutralize digestion and centrifuged at 1300 rpm for 10 minutes at 4ºC to pellet down the leukocytes. Cells were suspended in complete media (10% FBS containing RPMI 1640) and counted, used for flow cytometry staining.

Mouse model of Transverse Aortic constriction (TAC)

TAC surgery was performed as previously described.[6, 7] Briefly, mice were anesthetized with ketamine (55 mg/kg) plus xylazine (15 mg/kg). Animals were intubated and ventilated with room air with a mouse respirator (Harvard Apparatus, Holliston, MA). Rectal temperatures were maintained at 37°C by a thermo-regulated heating pad. After identifying the transverse aorta at surgery, a small piece of a 6.0 silk suture was placed between the innominate and left carotid arteries. Two loose knots were tied around the transverse aorta, and a small piece of a 27½ gauge blunt needle was placed parallel to the transverse aorta. The first knot was quickly tied against the needle, followed by the second, and the needle was promptly removed to yield a constriction of 0.4mm in diameter. In sham-operated control mice, the entire procedure was identical except for the ligation of the aorta. The mice were closely monitored until fully conscious.

Transthoracic echocardiography

Echocardiography was performed using the Vevo 3100 high-resolution ultrasound imaging system with a MX400 transducer (Visual Sonics Inc., Toronto CA). Mice were anesthetized with 1.5-2% isoflurane and placed on a temperature-maintained platform in a supine position. Warmed ultrasound transmission gel was placed on the chest (hair removed), and M-mode interrogation (for quantification of various cavity dimensions and wall thicknesses) was performed in the parasternal short-axis view at the level of the greatest LV end-diastolic dimension (LVEDD). Ejection fraction, fractional shortening, and other parameters were calculated using the Vevo 3100 software. Mice with heart rates lower than 400 bpm (beats per minute) were omitted from the analysis.

Tissue Processing, Histology, and Immunofluorescence assay

Animals were sacrificed under anesthesia after 4 weeks of ponatinib treatment, and their hearts were removed, formalin-fixed, and paraffin-embedded for histology following previously published protocols.[8, 9] Samples were stained with Masson's trichrome (Sigma-Aldrich; St. Louis, MO) for collagen deposition. Fibrosis was measured on Masson's trichrome-stained tissue sections using NIH Image J software. CD31 staining and TUNEL staining were performed as per previously published protocols.[10, 11] Fluorescent images were taken on a KEYENCE BZ-X800 fluorescence microscope. The representative images were chosen as per mean/average.

RNA sequencing

Total RNA was extracted from the ventricles of HFD-fed ApoE−/− mice treated with ponatinib and placebo controls (n=5/group) using the RNeasy Mini kit (74104, Qiagen) as

per the manufacturer's instructions. RNA integrity was confirmed using the Agilent Bioanalyzer. RNA sequencing (RNA-Seq) was performed by the Vanderbilt Technologies for Advanced Genomics (VANTAGE) core. RNA libraries were constructed using the Illumina TruSeq Stranded Total RNA kit. Libraries were sequenced using Illumina HiSeq 3000 on paired-end-75 flow cell runs at ~35M PF reads per sample. Raw reads (fastq files) were uploaded to the Partek Flow server and pre-alignment quality assessment was performed. Mean base-call quality scores were above Phred-like values of 36 in all positions of all samples, and no hard trimming of the reads was necessary. Sequences were aligned to the mm10 assembly of the mouse genome using STAR 2.5.3a. The resulting summary of reads quantified at the gene level to Ensemble transcripts 83 using Partek's expectation-maximization (E/M) annotation model. Gene counts were normalized to the total read count per sample and then log-transformed (with an offset of 0.0001).

To identify differentially expressed genes, statistical analysis was performed using Partek's Gene Specific Analysis (GSA) multimodel estimation algorithm, which identifies the statistical model that is the best for each gene among all the selected models (lognormal, Poisson, etc.), and then uses that best model to calculate p-value and fold change. Hierarchical clustering was performed on normalized and log-transformed counts using Partek Genomics Suite 7.19.1125. Statistical analyses (including correction for multiple hypothesis testing) for identification of overrepresented ontologies, functions, and pathways were performed using Ingenuity Pathway Analysis (Qiagen), as well as DAVID (https://david.ncifcrf.gov), a freely available online-based functional analysis tool to identify relevant gene sets.

Antibodies and Reagents

Following antibodies and reagents were used: APC-Cy™7 anti-Mouse CD45 (clone: 30-F11), PerCP Anti-Mouse CD45 (clone: 30-F11), PE-Cy™7Anti-Mouse CD3e (clone: 145-2C11), FITC Anti-Mouse CD4 (clone: RM4-5), PE-CF594 Anti-Mouse CD4 (clone: RM4-5), PE-CF594 Anti-Mouse TCR β Chain (clone: H57-597), PerCP-Cy™5.5 Anti-Mouse CD8a (clone: 53-6.7), BV421 Anti-Mouse IFN-γ (clone: XMG1.2), PE Anti-Mouse IL-4 (clone: 11B11), Alexa Fluor® 700 Anti-Mouse IL-17A (clone: TC11-18H10), Alexa Fluor® 647 anti-Mouse IL-9 (clone: D9302C12), PE-Cy™7 Anti-CD11b (clone: M1/70), FITC Anti-Mouse Ly-6C (clone: AL-21), BV421 Anti-Mouse F4/80 (clone: T45-2342), BV510 Rat Anti-Mouse Mer (clone: 108928), PE Anti-Mouse Ly-6G (clone: 1A8), APC Anti-Mouse Ly-6G and Ly-6C (clone: RB6-8C5), PE Anti-Mouse TNF(clone: MP6-XT22), Alexa Fluor® 488 Anti-Mouse IL-6 (clone: MP5-20F3), BV421 Anti-Mouse CD25 (clone: PC61), PE anti-Mouse Foxp3 (clone: MF23 and Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™) was from BD Pharmingen, USA. APC IL-1 beta anti-Mouse (clone: NJTEN3), Alexa Fluor® 700 anti-Mouse/Human Ki-67 (clone: SolA15), PerCP-Cyanine5.5 anti-Mouse CD11c (clone: N418), PE anti-Mouse CXCL9 (clone: MIG-2F5.5), PE anti-mouse CD284 (TLR4) (clone: UT41), APC anti-Mouse CD206 (clone: MR6F3) and Alexa Fluor 700 anti-Mouse MHC Class II (I-A/I-E) (clone: M5/114.15.2) was from eBioscience™, USA. Alexa Fluor 647 anti-Mouse CCL2/JE/MCP-1 (clone: 123616R), Alexa Fluor 750 anti-Mouse CCR2 (clone: 475301), Alexa Fluor 405 NLRP3/NALP3 anti-Mouse/Human (clone: 768319) was from R&D Systems™. IgG (H+L) Cross-Adsorbed F(ab ft.)2-Goat anti-Rabbit, PE, Invitrogen™. S100A8/A9 anti-Mouse/Human (clone: MRP8 7C12/4) Novus Biologicals. TNF alpha Mouse Uncoated ELISA Kit (catalog# 88-

7324-22), IL-6 Mouse Uncoated ELISA Kit (catalog# 88-7064-22) and IL-1 beta Mouse Uncoated ELISA Kit from Invitrogen™ (catalog# 88-7013-88).

Flow cytometry analyses

Mouse hearts were digested, and leukocytes were isolated and counted. Spleens were isolated from mice and macerated in RPMI 1640 (Gibco, Invitrogen, UK), containing 10% FBS to prepare a single-cell suspension. A single-cell suspension was made from BM of mice. Red blood cells (RBCs) were lysed with RBC lysis buffer (Quality Biological, Inc.), incubated at room temperature for 1 minute, and washed with 10% RPMI 1640. The cells were counted, and 0.5-1×106 cells were used for surface staining post Fc blocking (1µg/ml) in 3% FBS for 30 minutes over ice. For intracellular staining, 0.5-1×106 cells were cultured per well in 96-well plates (Nunc, USA) then incubated for 5 hrs in stimulating RPMI media containing Phorbol 12-myristate 13-acetate, or PMA, (0.1 mg/ml, Sigma), ionomycin (1 mg/ml, Sigma), and a 1:1000 dilution of Golgistop/Golgiplug (BD Biosciences). Following stimulation, cells were first surface stained for 30 minutes on ice. After washing with PBS, cells were fixed and permeabilized using the BD cytofix/cytoperm kit (BD Biosciences) for 30 minutes at 4°C, then washed again with BD perm wash kit (BD Biosciences) and stained with intracellular fluorescently labeled antibodies. Fluorescence intensity of fluorochrome-labeled cells was measured by flow cytometry (BD LSR-II) at UAB Flow Cytometry Core Facility. FACS Diva was used for acquiring the cells, and final data analysis was performed by Flow Jo (Tree star, USA). The representative images were chosen as per mean/average.

ImageStream Analysis

ImageStream cells were stained with PE-Cy™7 CD11b anti-Mouse (clone: M1/70, BD Pharmingen), Alexa Fluor 405 NLRP3/NALP3 anti-Mouse/Human (clone: 768319, R&D Systems™), IL-1β anti-Mouse (clone: NJTEN3, eBioscience™), and APC BrdU flow kit (cat:51-9000019AK, BD Pharmingen) for ImageStream analyses. Samples were imaged at 60× magnification while acquiring data on different channels. Single-color stained and unstained controls were used to calibrate the machine, calibration beads were used to adjust the noise levels and spectral compensation. Brightfield data were recorded on channels Ch01 and Ch09. 10000 events were acquired and used for analysis in IDEAS software version 6.2 (EMD Millipore, Billerica, MA, USA).

Quantitation of IL-1β, IL-6, TNF-α, and S100A8/9 by ELISA

Blood serum and supernatants from cell culture were assayed for mouse IL-1β (Invitrogen ref# 88-7013-22), IL-6 (Invitrogen ref# 88-7064-22), TNF-α (Invitrogen ref# 88-7324-22) and S100A8/9 heterodimer (R&D system cat# DY8596-05) according to the manufacturer's instructions.

***in vivo* proliferation assay**

Six hundred µg of BrdU in100µl of PBS was administered intraperitoneal to each mouse3 days prior to the experiment. Cells from both the heart tissue and spleen of mice were isolated and stained with anti-BrdU anti-body in the presence of DNase. Data were acquired by flow cytometry.

***in vitro* proliferation assay**

Flow sorted $CD11b^{+}$ cells from WT naive mice were labeled with CFSE (CellTrace CFSE, cat# C34554, from Invitrogen USA) and cultured for 72 hrs with 10 µg/ml LPS, Ponatinib

100nM, Ponatinib 100nM+ TLR-IN-C34 10μM, Ponatinib 100nM+ CY09 10μM, Ponatinib 100nM+ Paquinimod 0.5μM. The percentages of $CD11b^{+}CFSE^{low}$ cells (proliferating cells) were determined by FACS analysis. The representative images were chosen as per mean/average.

Glucose and Cholesterol assays

Serum glucose and cholesterol concentration levels were detected using a mouse glucose assay kit supplied by Crystal Chemical (cat# 81692) and cholesterol reagent with a standard kit (RAICHWM ref# R80035) according to the manufacturer's instructions.

Neonatal Rat Ventricular Fibroblasts culture and treatments

Neonatal rat ventricular fibroblasts (NRVFs) were isolated from 1-2-day-old Sprague-Dawley rats as described previously.[12] NRVFs were cultured in DMEM with 10% FBS for 24hrs. Cells were serum-starved for 16-18hs. After starvation cells were pre-treated with ponatinib (100nM, 250nM) for 1h followed by TGF-β1 treatment (10ng/ml, 1h).

Mass-spectromtery of plasma samples

Eight weeks old C57BL/6J mice were subjected to single dose of ponatinib 15mg/kg. Blood plasma was collected at 2hrs, 4hrs, 6hrs, 8hrs and 22 hrs. Plasma samples were submitted to Vanderbilt Mass-Spectrometry core for analysis.

NRVF isolation

Hearts were excised, rinsed in cold Hank's balanced salt solution, minced, and digested with type II collagenase (100U/mL) (Worthington) and pancreatin (0.6 mg/mL) (Sigma) at 37°C for 15 minutes. The first digestion was discarded. The collagenase medium from the second digestion containing the cardiac fibroblasts was centrifuged for 5 minutes at

3000rpm and resuspended in DMEM with 10% fetal bovine serum/1% antibiotic solution. The digestion was repeated until the digestion fluid became clear (5–6 times). Cells were plated in 100-mm dishes (Corning, NY) and allowed to attach for 1 hour before the first media change, which removed weakly adherent cells, including myocytes and endothelial cells. Fibroblasts were washed twice with Ca2+- and Mg2+-free PBS (Cellgro, Mediatech, Inc), cultured in DMEM with 10% FBS, trypsinized (Invitrogen), and passaged as required based on cellular confluence.

Figure S1

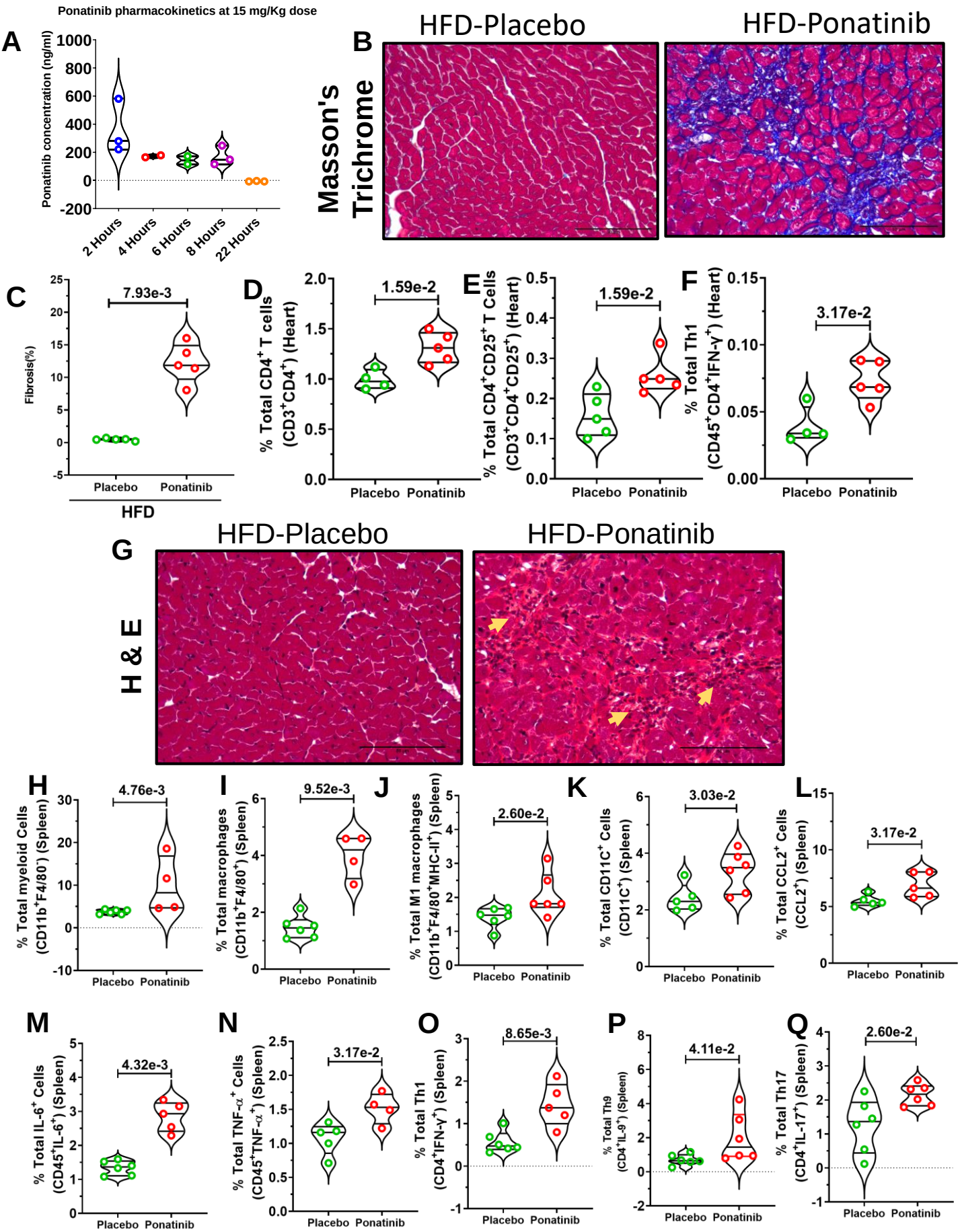

Ponatinib pharmacokinetics at 15 mg/Kg dose
A
Ponatinib concentration (ng/ml)
1000
800
600
400
200
0
-200
2 Hours
4 Hours
6 Hours
8 Hours
22 Hours
B
HFD-Placebo
HFD-Ponatinib
Masson's Trichrome
C
7.93e-3
Fibrosis(%)
25
20
15
10
5
0
-5
Placebo
Ponatinib
HFD
D
1.59e-2
% Total CD4+ T cells (CD3+CD4+) (Heart)
2.0
1.5
1.0
0.5
0.0
Placebo
Ponatinib
E
1.59e-2
% Total CD4+CD25+ T Cells (CD3+CD4+CD25+) (Heart)
0.5
0.4
0.3
0.2
0.1
0.0
Placebo
Ponatinib
F
3.17e-2
% Total Th1 (CD45+CD4+IFN-γ+) (Heart)
0.15
0.10
0.05
0.00
Placebo
Ponatinib
G
HFD-Placebo
HFD-Ponatinib
H & E
H
4.76e-3
% Total myeloid Cells (CD11b+F4/80+) (Spleen)
30
20
10
0
-10
Placebo
Ponatinib
I
9.52e-3
% Total macrophages (CD11b+F4/80+) (Spleen)
6
4
2
0
Placebo
Ponatinib
J
2.60e-2
% Total M1 macrophages (CD11b+F4/80+MHC-II+) (Spleen)
4
3
2
1
0
Placebo
Ponatinib
K
3.03e-2
% Total CD11C+ Cells (CD11C+) (Spleen)
6
4
2
0
Placebo
Ponatinib
L
3.17e-2
% Total CCL2+ Cells (CCL2+) (Spleen)
15
10
5
0
Placebo
Ponatinib
M
4.32e-3
% Total IL-6+ Cells (CD45+IL-6+) (Spleen)
5
4
3
2
1
0
Placebo
Ponatinib
N
3.17e-2
% Total TNF-α+ Cells (CD45+TNF-α+) (Spleen)
2.5
2.0
1.5
1.0
0.5
0.0
Placebo
Ponatinib
O
8.65e-3
% Total Th1 (CD4+IFN-γ+) (Spleen)
3
2
1
0
-1
Placebo
Ponatinib
P
4.11e-2
% Total Th9 (CD4+IL-9+) (Spleen)
8
6
4
2
0
-2
Placebo
Ponatinib
Q
2.60e-2
% Total Th17 (CD4+IL-17+) (Spleen)
4
3
2
1
0
-1
Placebo
Ponatinib

Figure S1: Ponatinib treatment induces adverse cardiac remodeling and promotes inflammation in HFD-fed ApoE-/- mice. (A) C57BL/6 mice were given 15mg/Kg of ponatinib once. Blood plasma was collected at 2 hours, 4 hours, 6 hours, 8 hours, and 22 hours time points. Plasma samples were subjected to mass spectrometry analysis to obtain ponatinib concentration (ng/ml). **(B-C)** Assessment of cardiac fibrosis by Masson's Trichrome staining **(B)** Trichrome-stained LV regions and **(C)** Quantification of LV fibrosis, placebo and ponatinib (N=5). **(D-F)** Quantitation of immune cells as a percentage of total cells (immune and nonimmune) isolated from the digested heart of HFD placebo and HFD ponatinib groups. (D) total percentage of CD4+ T cells (CD3+CD4+), placebo (N=4) and ponatinib (N=5), **(E)** total CD4+CD25+ T cells (CD3+CD4+CD25+), placebo (N=5) and ponatinib (N=5), **(F)** total percentage of Th1 (CD45+CD4+IFN-γ)+ T cells, placebo (N=4) and ponatinib (N=5). **(G)** Representative H&E-stained LV regions in HFD-fed placebo and ponatinib-treated mice. Yellow arrows show sites on infiltrated immune cells in the LV. Scale bar = 50 μm. **(H-Q)** Quantitation of immune cells as a percentage of total cells isolated from the spleen of HFD-fed placebo and ponatinib-treated mice. Data represents quantitation of percent of **(H)** total myeloid cells (CD45+CD11b+F4/80-), placebo (N=6), ponatinib (N=4), **(I)** total macrophages (CD11b+F4/80+), placebo (N=6), ponatinib (N=4), **(J)** total M1 macrophages (CD11b+F4/80+MHC-II+), (placebo (N=6), ponatinib (N=6), **(K)** total dendritic cells (CD11C +), placebo (N=5), ponatinib (N=6), **(L)** total CCL2 cells (CCL2+), placebo (N=5), ponatinib (N=5), **(M)** total IL-6 producing cells (CD45+IL-6+), placebo (N=6), ponatinib (N=5), (N) total TNF-α producing cells (CD45+TNF-α+), placebo (N=5), ponatinib (N=4, **(O)**) total Th1 cells (CD4+IFN-γ+), placebo (N=6), ponatinib (N=5), **(P)** total Th9 cell (CD4+IL-9+), placebo (N=6), ponatinib (N=6), and **(Q)** total Th17 cell (CD4+IL-17+), placebo (N=6), ponatinib (N=6). Data **(C-F, H-Q)** were analyzed using the Mann-Whitney U test and represented as mean±SEM.

Figure S2

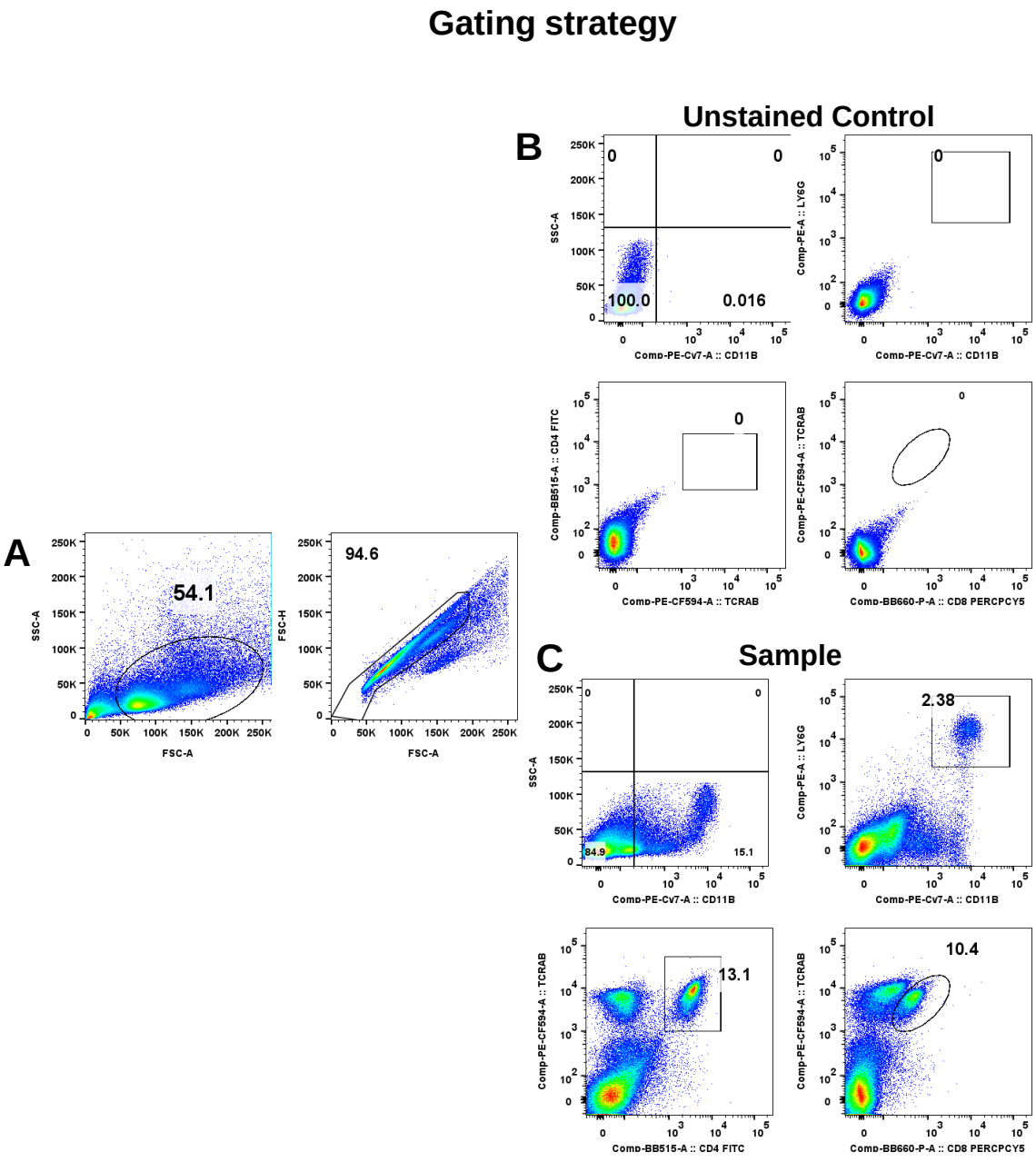


Figure S2: Gating strategy for flow cytometry analysis of immune cells (myeloid cells, neutrophils, CD4+tive T-cells, and CD8+tive T-cells.

Figure S3

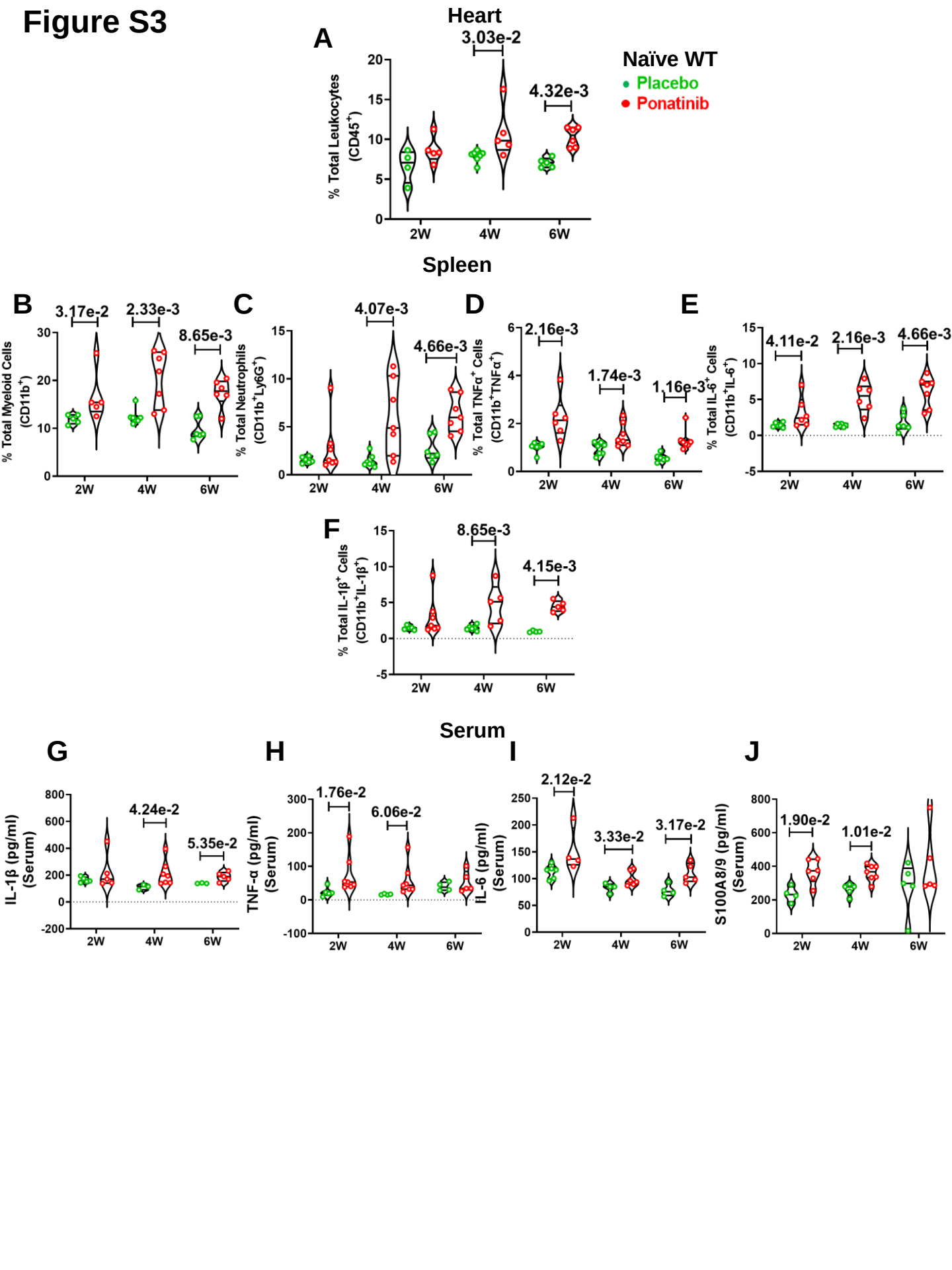

Heart
A
3.03e-2
4.32e-3
% Total Leukocytes (CD45+)
Naïve WT
Placebo
Ponatinib
2W
4W
6W
Spleen
B
3.17e-2
2.33e-3
8.65e-3
% Total Myeloid Cells (CD11b+)
C
4.07e-3
4.66e-3
% Total Neutrophils (CD11b+Ly6G+)
D
2.16e-3
1.74e-3
1.16e-3
% Total TNFα+ Cells (CD11b+TNFα+)
E
4.11e-2
2.16e-3
4.66e-3
% Total IL-6+ Cells (CD11b+IL-6+)
F
8.65e-3
4.15e-3
% Total IL-1β+ Cells (CD11b+IL-1β+)
Serum
G
4.24e-2
5.35e-2
IL-1β (pg/ml) (Serum)
H
1.76e-2
6.06e-2
TNF-α (pg/ml) (Serum)
I
2.12e-2
3.33e-2
3.17e-2
IL-6 (pg/ml) (Serum)
J
1.90e-2
1.01e-2
S100A8/9 (pg/ml) (Serum)

Figure S3: Ponatinib treatment promotes sustained chronic inflammation. At 10 weeks of age, C57BL/6J mice were subjected to ponatinib (15 mg/kg/day) treatment for 6 weeks. Quantitation of immune cells as a percentage of total cells **(A)** CD45+ leukocytes in heart, At 2 weeks, placebo (N=4), ponatinib (N=5); at 4 weeks, placebo (N=7), ponatinib (N=5); At 6 weeks, placebo (N=5), ponatinib (N=6), **(B)** myeloid cells (CD11b+), At 2 weeks, placebo (N=5), ponatinib (N=5); At 4 weeks, placebo (N=7), ponatinib (N=7); At 6 weeks, placebo (N=5), ponatinib (N=6), **(C)** neutrophils (CD11b+LY6G+), At 2 weeks, placebo (N=6), ponatinib (N=7); At 4 weeks, placebo (N=7), ponatinib (N=7); At 6 weeks, placebo (N=6), ponatinib (N=7), **(D)** myeloid cells expressing TNF-α (CD11b+TNF-α+), At 2 weeks, placebo (N=6), ponatinib (N=6); At 4 weeks, placebo (N=7), ponatinib (N=7); At 6 weeks, placebo (N=6), ponatinib (N=7), **(E)** myeloid cells expressing IL-6 (CD11b+IL-6+), At 2 weeks, placebo (N=6), ponatinib (N=6); At 4 weeks, placebo (N=6), ponatinib (N=6); At 6 weeks, placebo (N=6), ponatinib (N=7), **(F)** myeloid cells expressing IL-1b (CD11b+IL-1β+), At 2 weeks, placebo (N=5), ponatinib (N=7); At 4 weeks, placebo (N=6), ponatinib (N=5); At 6 weeks, placebo (N=4), ponatinib (N=5). **(G-J)** ELISA was performed from the serum obtained from animal groups. Data showing the concentration of **(G)** IL-1β, At 2 weeks, placebo (N=5), ponatinib (N=5); At 4 weeks, placebo (N=3), ponatinib (N=7); At 6 weeks, placebo (N=3), ponatinib (N=5), **(H)** TNF-α, At 2 weeks, placebo (N=5), ponatinib (N=7); At 4 weeks, placebo (N=4), ponatinib (N=7); At 6 weeks, placebo (N=4), ponatinib (N=5), **(I)** IL-6, At 2 weeks, placebo (N=5), ponatinib (N=4); At 4 weeks, placebo (N=4), ponatinib (N=6); At 6 weeks, placebo (N=4), ponatinib (N=5), and **(J)** S100A8/9 in serum, At 2 weeks, placebo (N=4), ponatinib (N=6); At 4 weeks, placebo (N=4), ponatinib (N=7); at 6 weeks, placebo (N=5), ponatinib (N=5). Data (A-J) were analyzed and significance was compared between Placebo vs Ponatinib for 2, 4 and 6 weeks separately by using the Mann-Whitney U test and represented as mean±SEM.

Figure S4

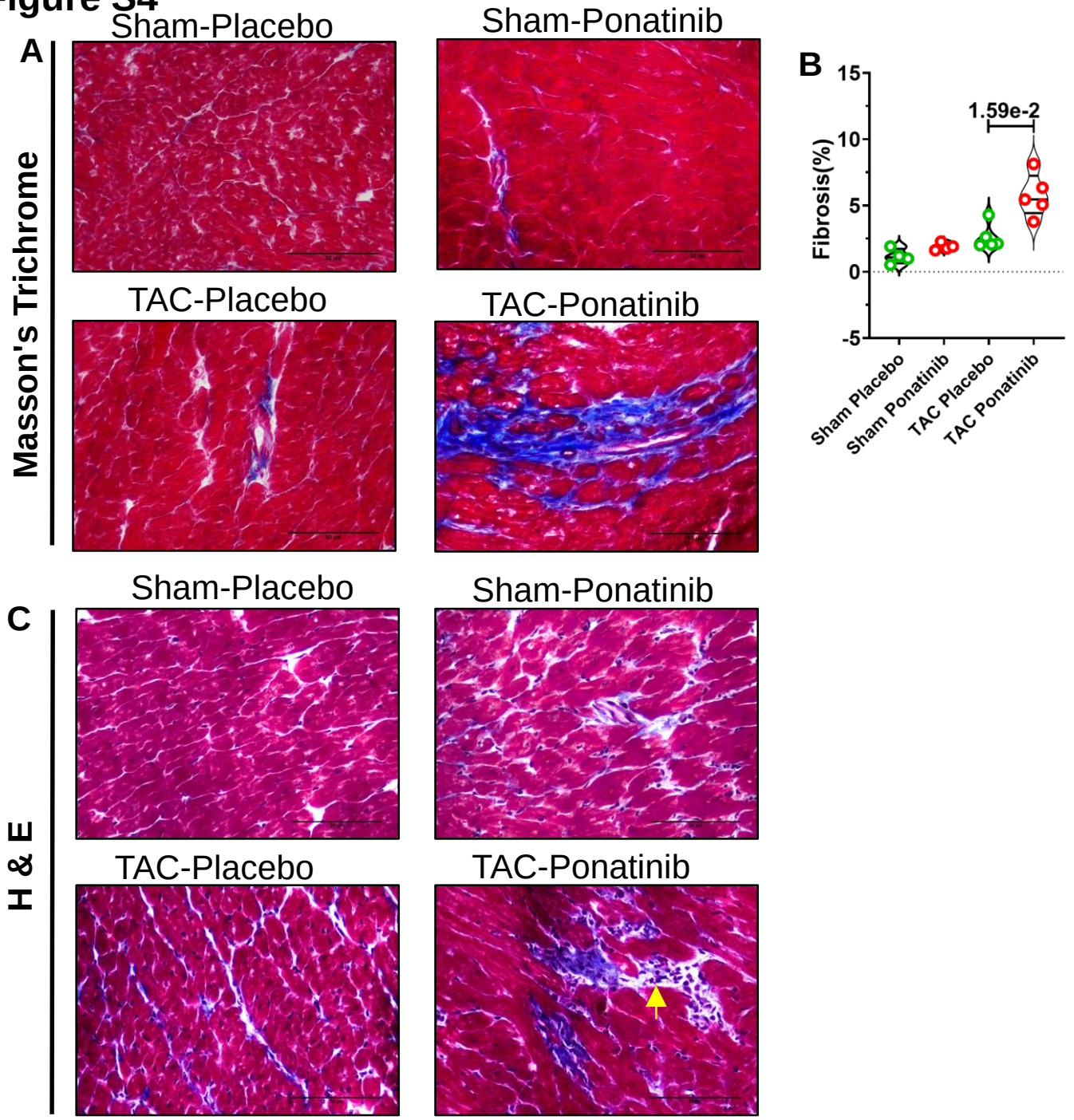


Figure S4: Ponatinib treatment induces cardiac remodeling ponatinib treated TAC mice. Assessment of immune cell infiltration (H&E) and cardiac fibrosis by Masson's Trichrome staining; **(A)** Trichrome-stained LV regions and, **(B)** Quantification of LV fibrosis, sham placebo (N=4), sham ponatinib (N=4), TAC placebo (N=5), TAC ponatinib (N=5), significance was compared between TAC placebo vs TAC ponatinib by using the Mann-Whitney U test and represented as mean±SEM.(C) Representative H&E-stained LV regions in Sham/TAC placebo and Sham/TAC-ponatinib treated mice. Yellow arrows show sites on infiltrated immune cells in the LV. Scale bar = 50 µm.

Figure S5

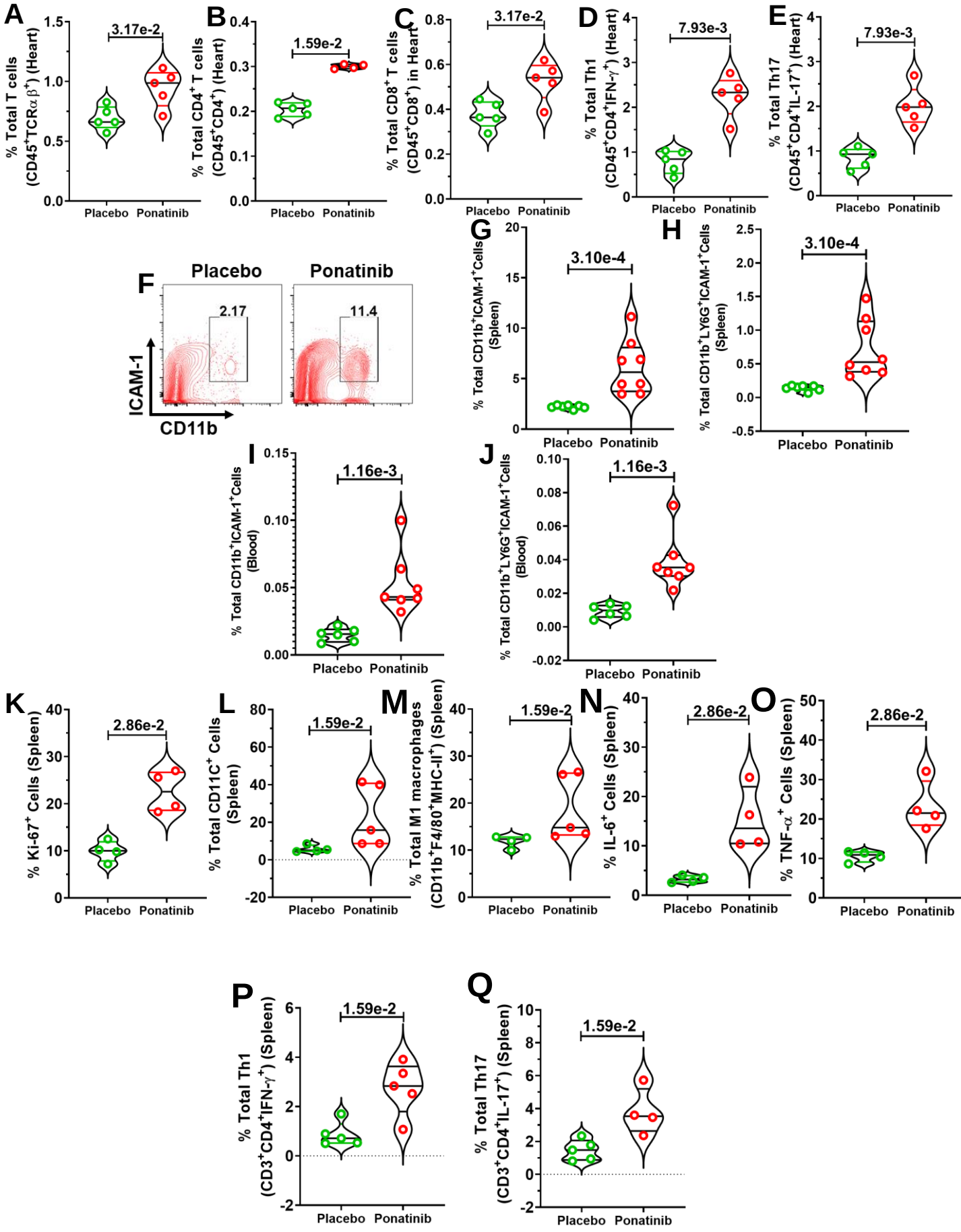

A
% Total T cells (CD45+TCRαβ+) (Heart)
3.17e-2
Placebo Ponatinib
B
% Total CD4+ T cells (CD45+CD4+) (Heart)
1.59e-2
Placebo Ponatinib
C
% Total CD8+ T cells (CD45+CD8+) in Heart
3.17e-2
Placebo Ponatinib
D
% Total Th1 (CD45+CD4+IFN-γ+) (Heart)
7.93e-3
Placebo Ponatinib
E
% Total Th17 (CD45+CD4+IL-17+) (Heart)
7.93e-3
Placebo Ponatinib
F
Placebo
Ponatinib
2.17
11.4
ICAM-1
CD11b
G
% Total CD11b+ICAM-1+Cells (Spleen)
3.10e-4
Placebo Ponatinib
H
% Total CD11b+LY6G+ICAM-1+Cells (Spleen)
3.10e-4
Placebo Ponatinib
I
% Total CD11b+ICAM-1+Cells (Blood)
1.16e-3
Placebo Ponatinib
J
% Total CD11b+LY6G+ICAM-1+Cells (Blood)
1.16e-3
Placebo Ponatinib
K
% Ki-67+ Cells (Spleen)
2.86e-2
Placebo Ponatinib
L
% Total CD11C+ Cells (Spleen)
1.59e-2
Placebo Ponatinib
M
% Total M1 macrophages (CD11b+F4/80+MHC-II+) (Spleen)
1.59e-2
Placebo Ponatinib
N
% IL-6+ Cells (Spleen)
2.86e-2
Placebo Ponatinib
O
% TNF-α+ Cells (Spleen)
2.86e-2
Placebo Ponatinib
P
% Total Th1 (CD3+CD4+IFN-γ+) (Spleen)
1.59e-2
Placebo Ponatinib
Q
% Total Th17 (CD3+CD4+IL-17+) (Spleen)
1.59e-2
Placebo Ponatinib

Figure S5: Ponatinib facilitates leukocytes' recruitment and enhances inflammation in ponatinib-treated TAC mice. (A-E) Quantitation of T cells as a percentage of total cells isolated from the heart of TAC-placebo and TAC-ponatinib groups. N=5 per group. Data show % total of **(A)** T cells (CD45+TCRαβ+), placebo and ponatinib (N=5), **(B)** CD4+ T cells (CD45+CD4+), placebo (N=5) and ponatinib (N=4), **(C)** CD8+ T cells (CD45+CD8+), placebo and ponatinib (N=5), **(D)** Th1 cells (CD45+CD4+IFN-γ+), placebo and ponatinib (N=5), and **(E)** Th17 cells (CD45+CD4+IL-17+), placebo and ponatinib (N=5). **(F)** Representative figure of flow cytometry showing gating strategy to measure ICAM-1 expressing immune cells. Data represents quantitation of percent of **(G)** total ICAM-1 expressing myeloid cells ($CD11b^+ICAM\text{-}1^+$), placebo (N=7), ponatinib (N=8), **(H)** total ICAM-1 expressing neutrophils ($CD11b^+LY6G^+ICAM\text{-}1^+$) in the spleen, placebo (N=7), ponatinib (N=8), and (**I)** total ICAM-1 expressing myeloid cells ($CD11b^+ICAM\text{-}1^+$), placebo (N=6), ponatinib (N7), **(J)** total ICAM-1 expressing neutrophils ($CD11b^+LY6G^+ICAM\text{-}1^+$) in the blood of TAC placebo and TAC ponatinib treated mice, placebo (N=6), ponatinib (N=7). **(K-Q)** Quantitation of immune cells as a percentage of total cells isolated from the spleen of TAC-placebo and TAC-ponatinib groups. Data represents quantitation of percent of **(K)** total proliferating cells ($Ki67^+$), placebo (N=4), ponatinib (N=4), **(L)** total dendritic cells ($CD11C^+$), placebo (N=4), ponatinib (N=5), **(M)** total M1 macrophages ($CD11b^+F4/80^+MHC\text{-}II^+$), placebo (N=4), ponatinib (N5), **(N)** total IL-6 producing cells ($IL\text{-}6^+$), placebo (N=4), ponatinib (N=4), **(O)** total TNF-α producing cells ($TNF\text{-}\alpha^+$), placebo (N=4), ponatinib (N=4), **(P)** total Th1 cells ($CD3^+CD4^+IFN\text{-}\gamma^+$), placebo (N=5), ponatinib (N=5), and **(Q)** total Th17 cells ($CD3^+CD4^+IL\text{-}17^+$), placebo (N=5), ponatinib (N4). Data **(A-Q)** were analyzed using the Mann-Whitney U test and represented as mean±SEM.

Figure S6

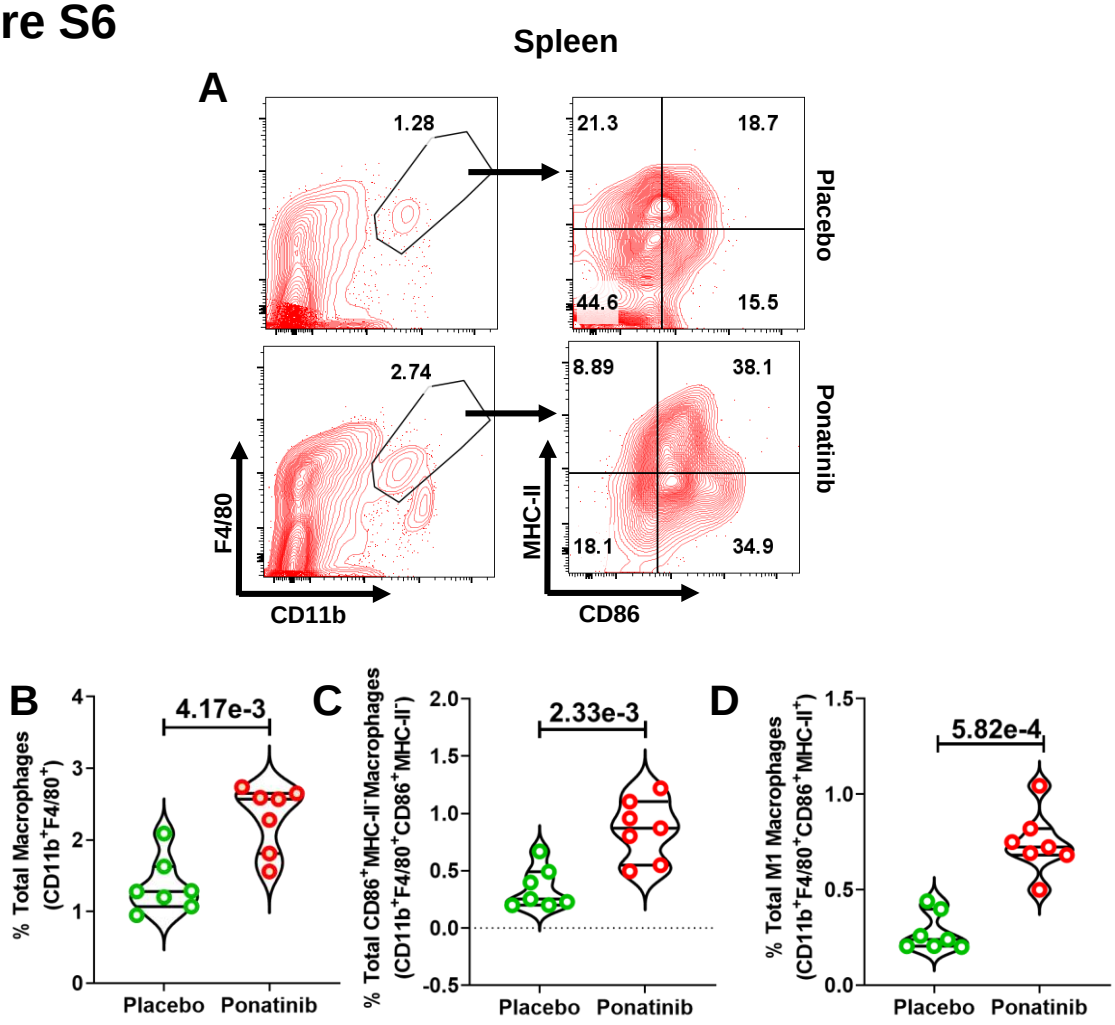


Figure S6| Ponatinib treatment induces macrophage population in the spleen of TAC mice. (A) Representative figure of flow cytometry showing gating strategy to measure M1 macrophage subsets from the spleen of TAC-placebo and TAC-ponatinib groups. Data represents quantitation of percent of total **(B)** macrophages ($CD11b^+F4/80^+$), **(C)** $CD11b^+F4/80^+CD86^+MHCII^-$ macrophages and, **(D)** M1 macrophages ($CD11b^+F4/80^+CD86^+MHC\text{-}II^+$). N=7 per group. Data (B-D) were analyzed using the Mann-Whitney U test and represented as mean±SEM.

Figure S7

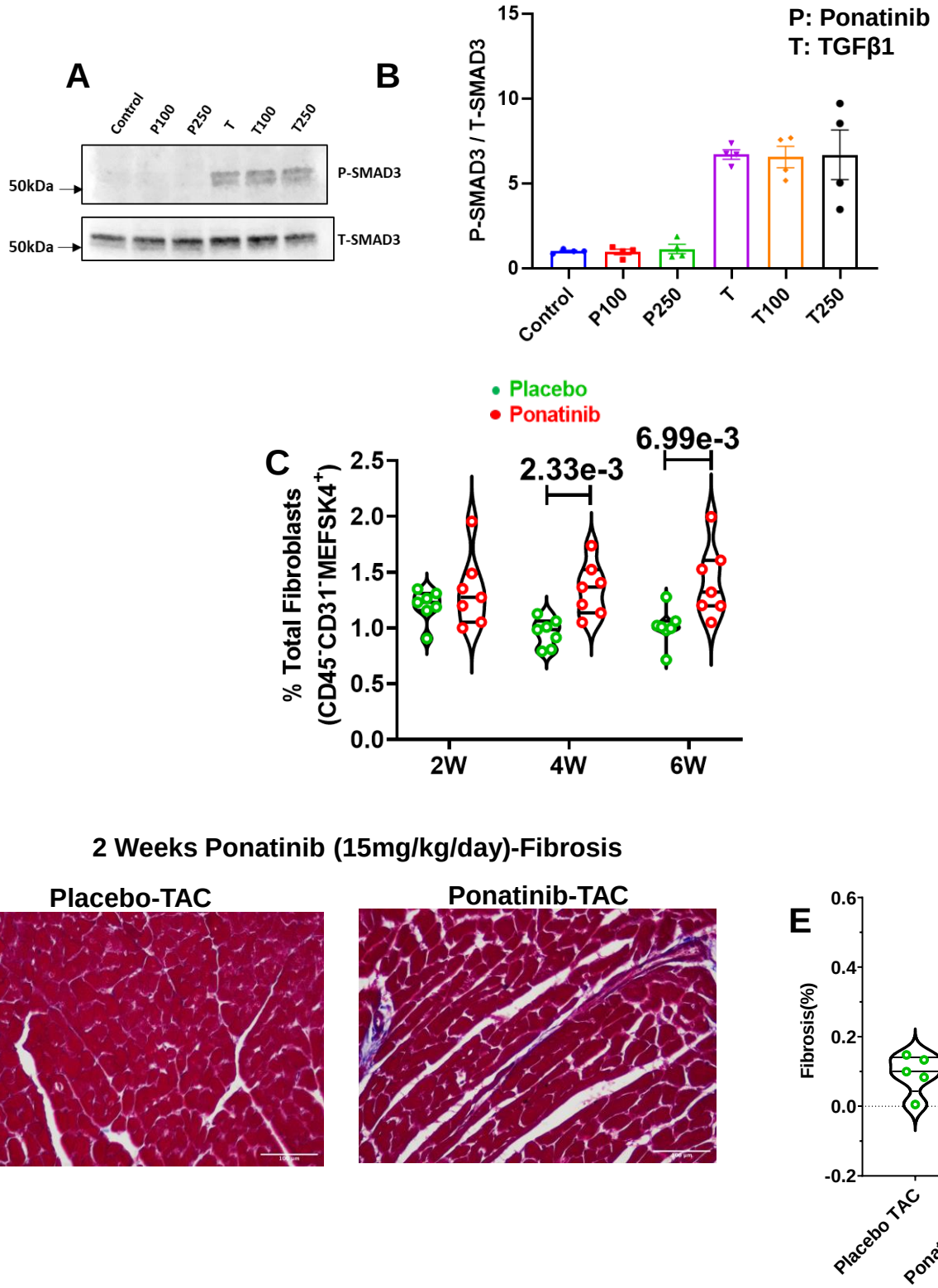

A
Control
P100
P250
T
T100
T250
50kDa
P-SMAD3
50kDa
T-SMAD3
B
P: Ponatinib
T: TGFβ1
P-SMAD3 / T-SMAD3
15
10
5
0
Control
P100
P250
T
T100
T250
C
Placebo
Ponatinib
% Total Fibroblasts (CD45-CD31-MEFSK4+)
2.5
2.0
1.5
1.0
0.5
0.0
2.33e-3
6.99e-3
2W
4W
6W
2 Weeks Ponatinib (15mg/kg/day)-Fibrosis
D
Placebo-TAC
Ponatinib-TAC
E
Fibrosis(%)
0.6
0.4
0.2
0.0
-0.2
Placebo TAC
Ponatinib TAC

Figure S7: Ponatinib treatment does not affect fibrosis at early time points. (A-B) Neonatal rat cardiac fibroblasts (CFs) were pretreated with ponatinib (100 nM and 500 nM) for 1 h followed by agonists treatment (10 ng/ml TGFβ1 for 1 h. Proteins were extracted, and Western blot analysis was carried out. Representative blots and Quantification. N=4 Data were analyzed using Kruskal-Wallis followed by Dunn test and represented as mean ± SEM **(C)** Representative violin plot showing total percentage on cardiac fibroblasts from ponatinib (15 mg/Kg/day) treated naïve mice at 2-, 4- and 6-weeks' time points. N=7 for each group of animals at all time points. Data were analyzed and significance was compared between Placebo vs Ponatinib for 2, 4 and 6 weeks separately by using the Mann-Whitney U test and represented as mean±SEM. **(D)** Masson's Trichrome staining in stained LV regions post 2 weeks treatment of ponatinib (15 mg/Kg/day) in TAC mice and **(E)** Quantification of LV fibrosis. Scale bar: 100μM. Placebo (N=5), ponatinib (N=6),Data were analyzed by using the Mann-Whitney U test and represented as mean±SEM.

Figure S8

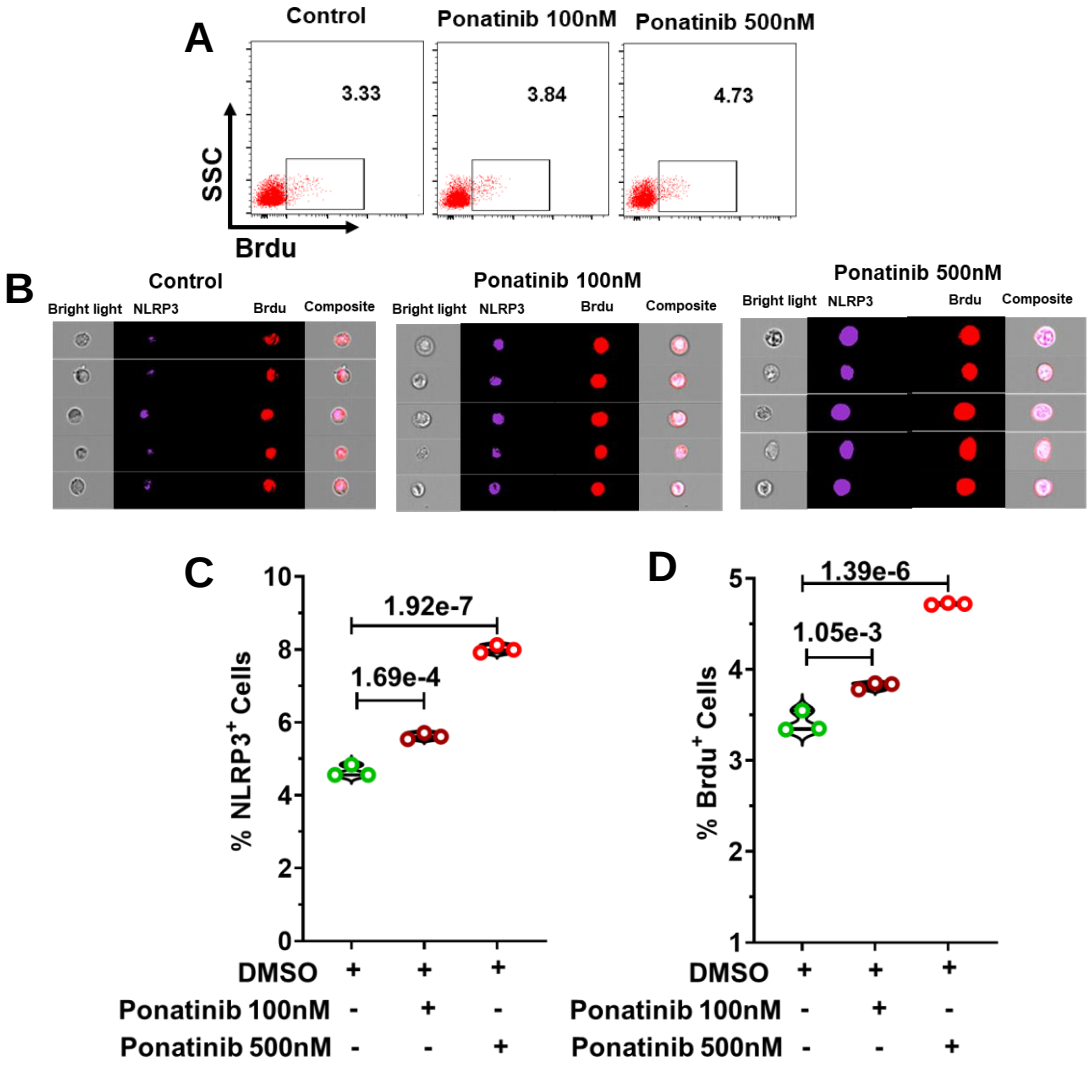


Figure S8: Ponatinib promotes proliferation in human PBMCs. (A-D) Human PBMCs were seeded with vehicle (DMSO) and ponatinib (100 nM and 500 nM, 72h). Cells were then incorporated with BrdU and flow cytometry and ImageStream analysis were performed. **(A)** Representative flow cytometry diagram showing ponatinib treatment promotes proliferation. **(B)** Representative ImageStream pictures showing ponatinib treatment upregulates NLRP3 expression and promotes proliferation in human PBMCs. **(C)** Total percentage of NLRP3+ cells. **(D)** Total percentage of proliferated (Brdu+) cells. Data **(C-D)** were analyzed by using ordinary one way ANOVA followed by Tukey's multiple comparisions test and represented as mean±SEM.

Figure S9

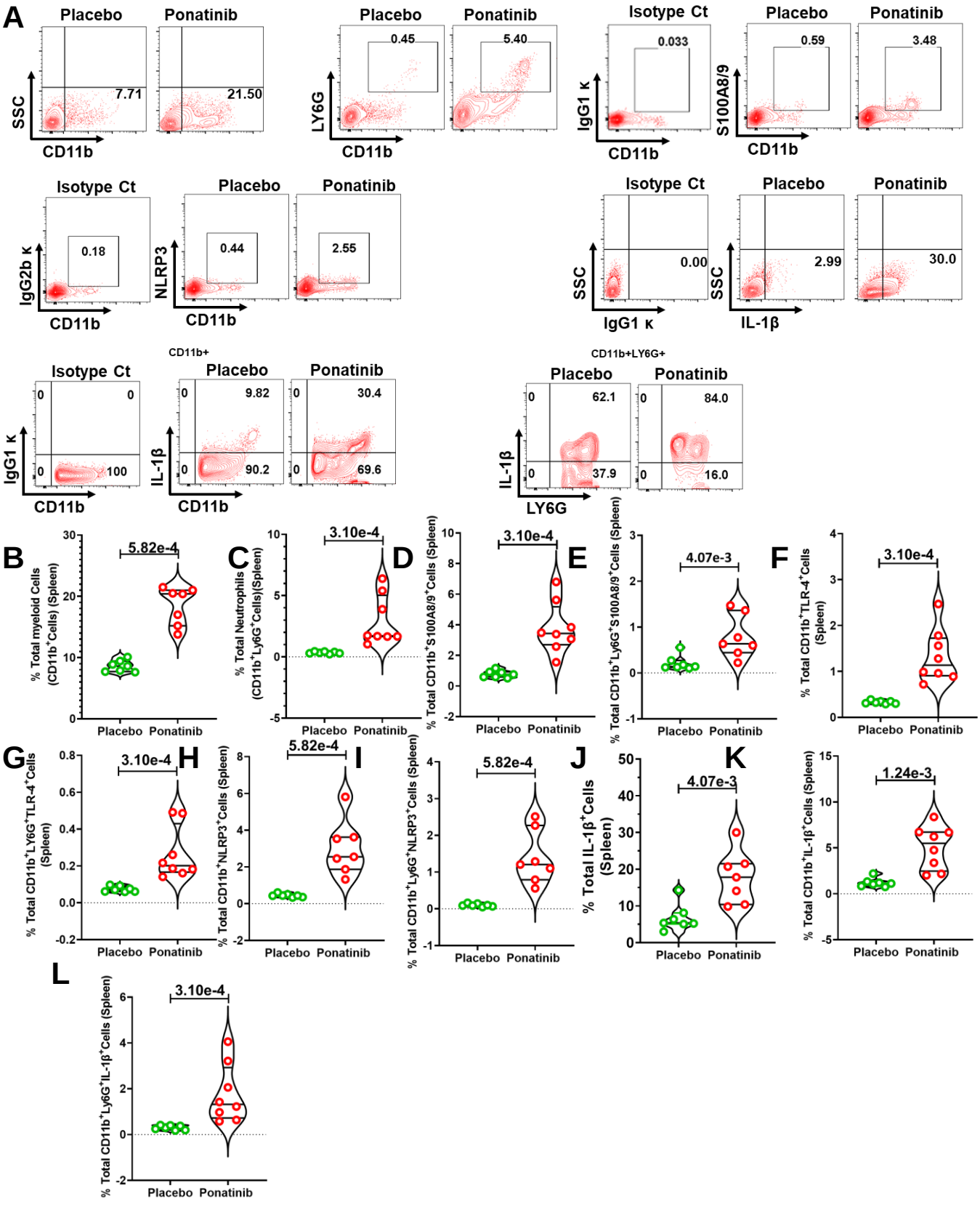

Spleen
A
Placebo
Ponatinib
SSC
CD11b
7.71
21.50
LY6G
0.45
5.40
Isotype Ct
IgG1 κ
0.033
S100A8/9
0.59
3.48
IgG2b κ
0.18
NLRP3
0.44
2.55
0.00
IL-1β
2.99
30.0
CD11b+
CD11b+LY6G+
0
100
9.82
90.2
30.4
69.6
62.1
37.9
84.0
16.0
LY6G
B
% Total myeloid Cells (CD11b+Cells) (Spleen)
5.82e-4
C
% Total Neutrophils (CD11b+Ly6G+Cells)(Spleen)
3.10e-4
D
% Total CD11b+S100A8/9+Cells (Spleen)
3.10e-4
E
% Total CD11b+Ly6G+S100A8/9+Cells (Spleen)
4.07e-3
F
% Total CD11b+TLR-4+Cells (Spleen)
3.10e-4
G
% Total CD11b+LY6G+TLR-4+Cells (Spleen)
3.10e-4
H
% Total CD11b+NLRP3+Cells (Spleen)
5.82e-4
I
% Total CD11b+Ly6G+NLRP3+Cells (Spleen)
5.82e-4
J
% Total IL-1β+Cells (Spleen)
4.07e-3
K
% Total CD11b+IL-1β+Cells (Spleen)
1.24e-3
L
% Total CD11b+Ly6G+IL-1β+Cells (Spleen)
3.10e-4
Placebo
Ponatinib

Figure S9: Ponatinib promotes inflammation through the NLRP3 pathway in ponatinib-treated TAC mice <u>(Data from Spleen).</u> (A) Representative figure of flow cytometry showing gating strategy to measure S100A8/9, NLRP3 and IL-1β expressing immune cells from the spleen of TAC-placebo and TAC-ponatinib groups. Data represents quantitation of percent of total **(B)** myeloid cells ($CD11b^+$), placebo and ponatinib (N=7), **(C)** neutrophils ($CD11b^+LY6G^+$) placebo (N=7), ponatinib (N=8), **(D)** S100A8/9 producing myeloid cells ($CD11b^+S100A8/9^+$), placebo (N=7), ponatinib (N=8), **(E)** S100A8/9 producing neutrophils ($CD11b^+LY6G^+S100A8/9^+$), placebo and ponatinib (N=7), **(F)** TLR-4 producing myeloid cells ($CD11b^+TLR\text{-}4^+$), placebo (N=7), ponatinib (N=8), **(G)** TLR-4 producing neutrophils ($CD11b^+LY6G^+TLR\text{-}4^+$), placebo (N=7), ponatinib (N=8), **(H)** NLRP3 producing myeloid cells ($CD11b^+NLRP3^+$), placebo and ponatinib (N=7), **(I)** NLRP3 producing neutrophils ($CD11b^+LY6G^+NLRP3^+$), placebo and ponatinib (N=7), **(J)** IL-1β producing cells, **(K)** IL-β producing myeloid cells ($CD11b^+IL\text{-}1\beta^+$), placebo and ponatinib (N=7), and **(L)** IL-β producing neutrophils ($CD11b^+LY6G^+$ IL-β^+), placebo (N=7), ponatinib (N=8). Data **(B-L)** were analyzed by using the Mann-Whitney U test and represented as mean±SEM.

Figure S10

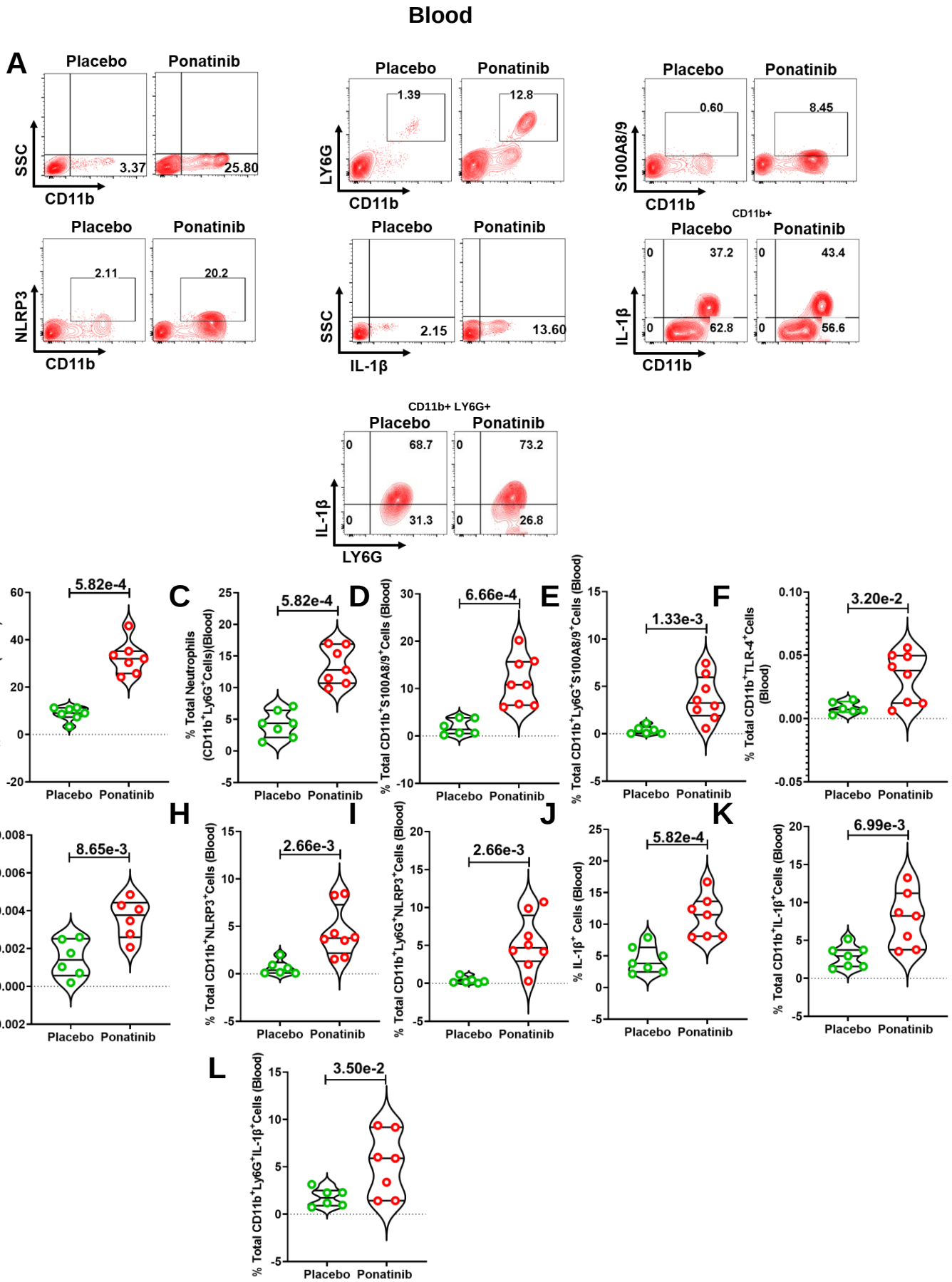

Blood
A
Placebo
Ponatinib
SSC
CD11b
3.37
25.80
LY6G
1.39
12.8
S100A8/9
0.60
8.45
NLRP3
2.11
20.2
IL-1β
2.15
13.60
CD11b+
0
37.2
62.8
43.4
56.6
CD11b+ LY6G+
68.7
31.3
73.2
26.8
B
% CD11b+Cells (Blood)
5.82e-4
C
% Total Neutrophils (CD11b+Ly6G+Cells)(Blood)
5.82e-4
D
% Total CD11b+S100A8/9+Cells (Blood)
6.66e-4
E
% Total CD11b+Ly6G+S100A8/9+Cells (Blood)
1.33e-3
F
% Total CD11b+TLR-4+Cells (Blood)
3.20e-2
G
% Total CD11b+LY6G+TLR-4+Cells (Blood)
8.65e-3
H
% Total CD11b+NLRP3+Cells (Blood)
2.66e-3
I
% Total CD11b+Ly6G+NLRP3+Cells (Blood)
2.66e-3
J
% IL-1β+ Cells (Blood)
5.82e-4
K
% Total CD11b+IL-1β+Cells (Blood)
6.99e-3
L
% Total CD11b+Ly6G+IL-1β+Cells (Blood)
3.50e-2
Placebo
Ponatinib

Figure S10: Ponatinib promotes inflammation through the NLRP3 pathway in ponatinib-treated TAC mice (Data from Blood). (A) Representative figure of flow cytometry showing gating strategy to measure S100A8/9, NLRP3 and IL-1β positive immune cells from the blood of TAC-placebo and TAC-ponatinib groups. Data represents quantitation of percent of total **(B)** myeloid cells ($CD11b^+$), placebo and ponatinib (N=7), **(C)** neutrophils ($CD11b^+LY6G^+$), placebo and ponatinib (N=7), **(D)** S100A8/9 producing myeloid cells ($CD11b^+S100A8/9^+$), placebo (N=6), ponatinib (N=8), **(E)** S100A8/9 producing neutrophils ($CD11b^+LY6G^+S100A8/9^+$), placebo (N=6), ponatinib (N=8), **(F)** TLR-4 expressive myeloid cells ($CD11b^+TLR\text{-}4^+$), placebo (N=6), ponatinib (N=8), **(G)** TLR-4 expressive neutrophils ($CD11b^+LY6G^+TLR\text{-}4^+$), placebo and ponatinib (N=6), **(H)** $NLRP3^+$ myeloid cells ($CD11b^+NLRP3^+$), placebo (N=6), ponatinib (N=8), **(I)** NLRP3 + neutrophils ($CD11b^+LY6G^+NLRP3^+$), placebo (N=6), ponatinib (N=8), **(J)** IL-1β producing cells, placebo and ponatinib (N=7), **(K)** IL-β producing myeloid cells ($CD11b^+IL\text{-}1\beta^+$), placebo and ponatinib (N=7), and **(L)** IL-β producing neutrophils ($CD11b^+LY6G^+$ IL-β^+), placebo (N=6), ponatinib (N=7). Data **(B-L)** were analyzed by using the Mann-Whitney U test and represented as mean±SEM.

Figure S11

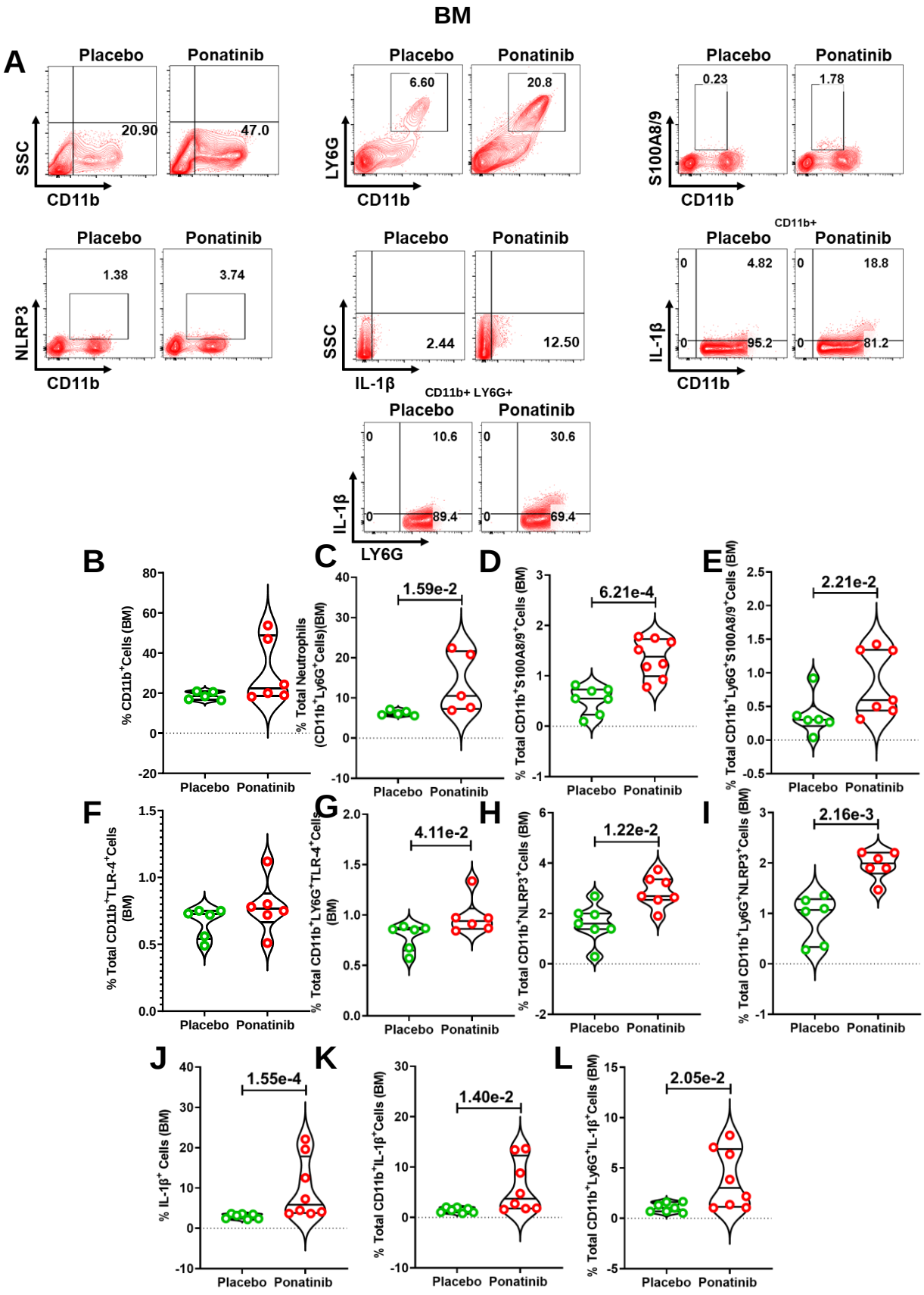

BM
A
Placebo
Ponatinib
SSC
CD11b
20.90
47.0
LY6G
6.60
20.8
S100A8/9
0.23
1.78
NLRP3
1.38
3.74
IL-1β
2.44
12.50
CD11b+
4.82
18.8
95.2
81.2
CD11b+ LY6G+
10.6
30.6
89.4
69.4
LY6G
B
% CD11b+Cells (BM)
C
% Total Neutrophils (CD11b+Ly6G+Cells)(BM)
1.59e-2
D
% Total CD11b+S100A8/9+Cells (BM)
6.21e-4
E
% Total CD11b+Ly6G+S100A8/9+Cells (BM)
2.21e-2
F
% Total CD11b+TLR-4+Cells (BM)
G
% Total CD11b+Ly6G+TLR-4+Cells (BM)
4.11e-2
H
% Total CD11b+NLRP3+Cells (BM)
1.22e-2
I
% Total CD11b+Ly6G+NLRP3+Cells (BM)
2.16e-3
J
% IL-1β+ Cells (BM)
1.55e-4
K
% Total CD11b+IL-1β+Cells (BM)
1.40e-2
L
% Total CD11b+Ly6G+IL-1β+Cells (BM)
2.05e-2

Figure S11: Ponatinib promotes inflammation through the NLRP3 pathway in ponatinib-treated TAC mice (Data from Bone marrow cells). (A) Representative figure of flow cytometry showing gating strategy to measure S100A8/9, NLRP3 and IL-1β expressing immune cells from the bone marrow of TAC-placebo and TAC-ponatinib groups. Data represents quantitation of percent of total **(B)** myeloid cells ($CD11b^+$), placebo (N=5), ponatinib (N=6), **(C)** neutrophils ($CD11b^+LY6G^+$), placebo and ponatinib (N=5), **(D)** S100A8/9 producing myeloid cells ($CD11b^+S100A8/9^+$), placebo (N=7), ponatinib (N=8), **(E)** S100A8/9 producing neutrophils ($CD11b^+LY6G^+S100A8/9^+$), placebo (N=6), ponatinib (N=7), **(F)** TLR-4 expressing myeloid cells ($CD11b^+TLR\text{-}4^+$), placebo and ponatinib (N=6), **(G)** TLR-4 expressing neutrophils ($CD11b^+LY6G^+TLR\text{-}4^+$), placebo and ponatinib (N=6), **(H)** NLRP3 expressing myeloid cells ($CD11b^+NLRP3^+$), placebo and ponatinib (N=7), **(I)** $NLRP3^+$ neutrophils ($CD11b^+LY6G^+NLRP3^+$), placebo and ponatinib (N=6), **(J)** IL-1β producing cells, placebo (N=7), ponatinib (N=8), **(K)** IL-β producing myeloid cells ($CD11b^+IL\text{-}1\beta^+$), placebo (N=7), ponatinib (N=8), and **(L)** IL-β producing neutrophils ($CD11b^+LY6G^+$ IL-β^+), placebo (N=7), ponatinib (N=8). Data **(B-L)** were analyzed by using the Mann-Whitney U test and represented as mean±SEM.

Figure S12

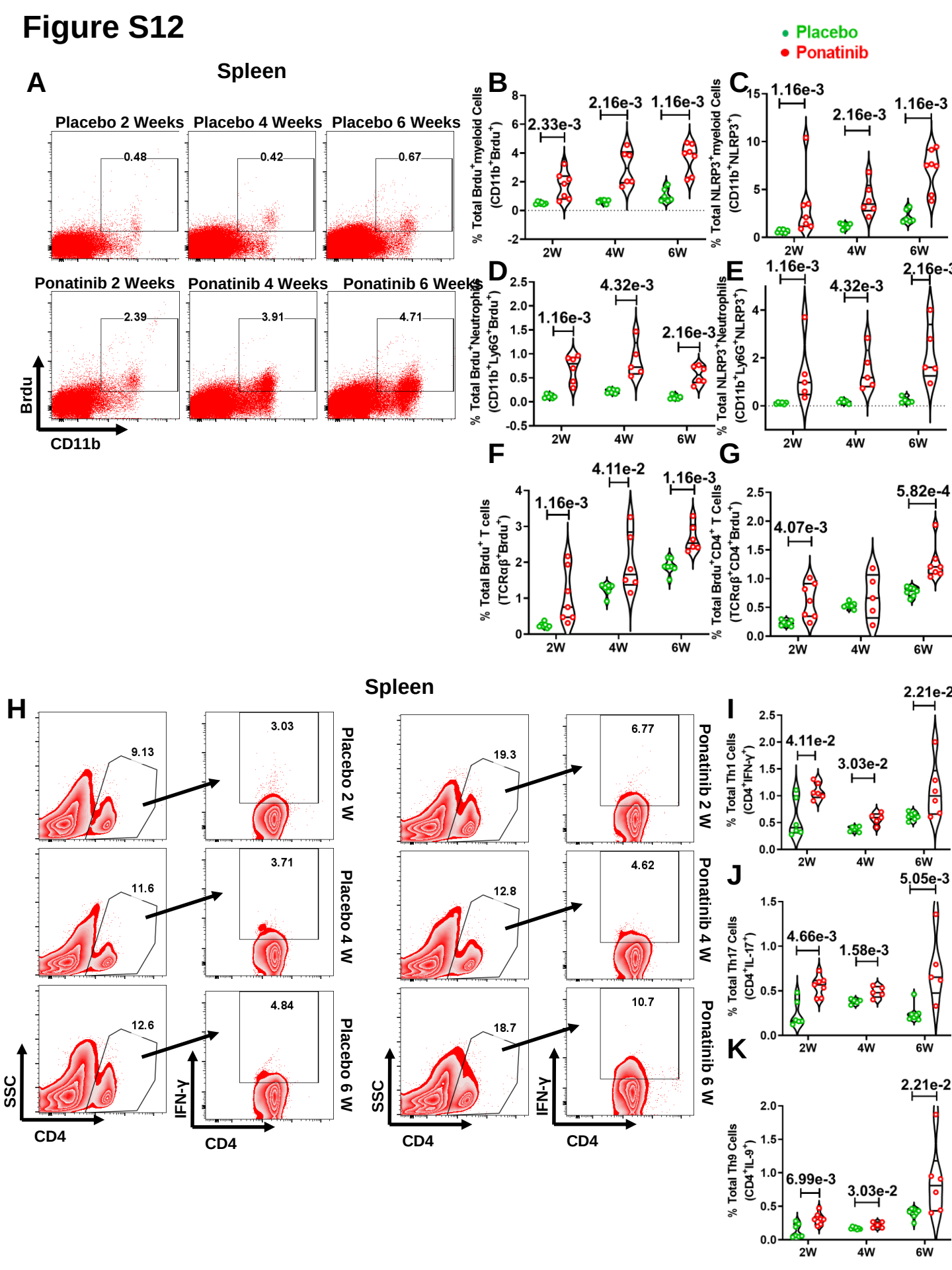

Placebo
Ponatinib
A
Spleen
Placebo 2 Weeks
Placebo 4 Weeks
Placebo 6 Weeks
0.48
0.42
0.67
Ponatinib 2 Weeks
Ponatinib 4 Weeks
Ponatinib 6 Weeks
2.39
3.91
4.71
Brdu
CD11b
B
% Total Brdu+myeloid Cells (CD11b+Brdu+)
2.33e-3
2.16e-3
1.16e-3
C
% Total NLRP3+myeloid Cells (CD11b+NLRP3+)
1.16e-3
2.16e-3
1.16e-3
D
% Total Brdu+Neutrophils (CD11b+Ly6G+Brdu+)
1.16e-3
4.32e-3
2.16e-3
E
% Total NLRP3+Neutrophils (CD11b+Ly6G+NLRP3+)
1.16e-3
4.32e-3
2.16e-3
F
% Total Brdu+ T cells (TCRαβ+Brdu+)
1.16e-3
4.11e-2
1.16e-3
G
% Total Brdu+CD4+ T Cells (TCRαβ+CD4+Brdu+)
4.07e-3
5.82e-4
2W
4W
6W
Spleen
H
9.13
3.03
Placebo 2 W
11.6
3.71
Placebo 4 W
12.6
4.84
Placebo 6 W
19.3
6.77
Ponatinib 2 W
12.8
4.62
Ponatinib 4 W
18.7
10.7
Ponatinib 6 W
SSC
CD4
IFN-γ
CD4
I
% Total Th1 Cells (CD4+IFN-γ+)
4.11e-2
3.03e-2
2.21e-2
J
% Total Th17 Cells (CD4+IL-17+)
4.66e-3
1.58e-3
5.05e-3
K
% Total Th9 Cells (CD4+IL-9+)
6.99e-3
3.03e-2
2.21e-2

Figure S12: Ponatinib promotes the proliferation of immune cells in naïve mice. At 10 weeks of age, C57BL/6J mice were subjected to ponatinib (15 mg/kg/day) treatment for 6 weeks. Immune cells were obtained from the spleens of these mice at 2,4- and 6 weeks post-treatment. Mice were given BrdU injections for 3 days (600µg / 100µg PBS) prior to harvesting. **(A)** Representative figure of flow cytometry showing gating strategy to measure proliferating $CD11b^+$ Cells from the spleen of placebo and ponatinib groups. Data represents quantitation of percent of total **(B)** proliferating myeloid cells ($CD11b^+Brdu^{+)}$, At 2 weeks, placebo (N=6), ponatinib (N=7); At 4 weeks, placebo (N=6), ponatinib (N=6); At 6 weeks, placebo (N=6), ponatinib (N=7), **(C)** $NLRP3^+$ myeloid cells ($CD11b^+NLRP3^+$), At 2 weeks, placebo (N=6), ponatinib (N=7); At 4 weeks, placebo (N=6), ponatinib (N=6); At 6 weeks, placebo (N=6), ponatinib (N=7), **(D)** proliferating neutrophils ($CD11b^+LY6G^+Brdu^{+)}$, At 2 weeks, placebo (N=6), ponatinib (N=6); At 4 weeks, placebo (N=6), ponatinib (N=5); At 6 weeks, placebo (N=6), ponatinib (N=6), **(E)** $NLRP3^+$ neutrophils ($CD11b^+LY6G^+NLRP3^+$), At 2 weeks, placebo (N=6), ponatinib (N=5); At 4 weeks, placebo (N=5), ponatinib (N=5); At 6 weeks, placebo (N=5), ponatinib (N=5), **(F)** proliferating T cells ($TCR\alpha\beta^+Brdu^+$), At 2 weeks, placebo (N=7), ponatinib (N=7); At 4 weeks, placebo (N=6), ponatinib (N=6); At 6 weeks, placebo (N=7), ponatinib (N=6), and **(G)** proliferating $CD4^+$ T cells ($TCR\alpha\beta^+CD4^+Brdu^+$), At 2 weeks, placebo (N=7), ponatinib (N=7); At 4 weeks, placebo (N=7), ponatinib (N=5); At 6 weeks, placebo (N=7), ponatinib (N=7). **(H)** Gating strategy to measure Th1 cell subsets. Data represents quantitation of percent of total **(I)** Th1 cells ($CD4^+IFN\text{-}\gamma^+$), At 2 weeks, placebo (N=6), ponatinib (N=6); At 4 weeks, placebo (N=6), ponatinib (N=5); At 6 weeks, placebo (N=7), ponatinib (N=6), **(J)** Th17 cells ($CD4^+IL\text{-}17^+$), At 2 weeks, placebo (N=6), ponatinib (N=7); At 4 weeks, placebo (N=5), ponatinib (N=5); At 6 weeks, placebo (N=7), ponatinib (N=5), and **(K)** Th9 cells ($CD4^+IL\text{-}9$), At 2 weeks, placebo (N=7), ponatinib (N=7); At 4 weeks, placebo (N=7), ponatinib (N=5); At 6 weeks, placebo (N=7), ponatinib (N=6). Data **(B-G and I-K)** were analyzed and significance was compared between Placebo vs Ponatinib for 2, 4 and 6 weeks separately by using the Mann-Whitney U test and represented as mean±SEM

Figure S13

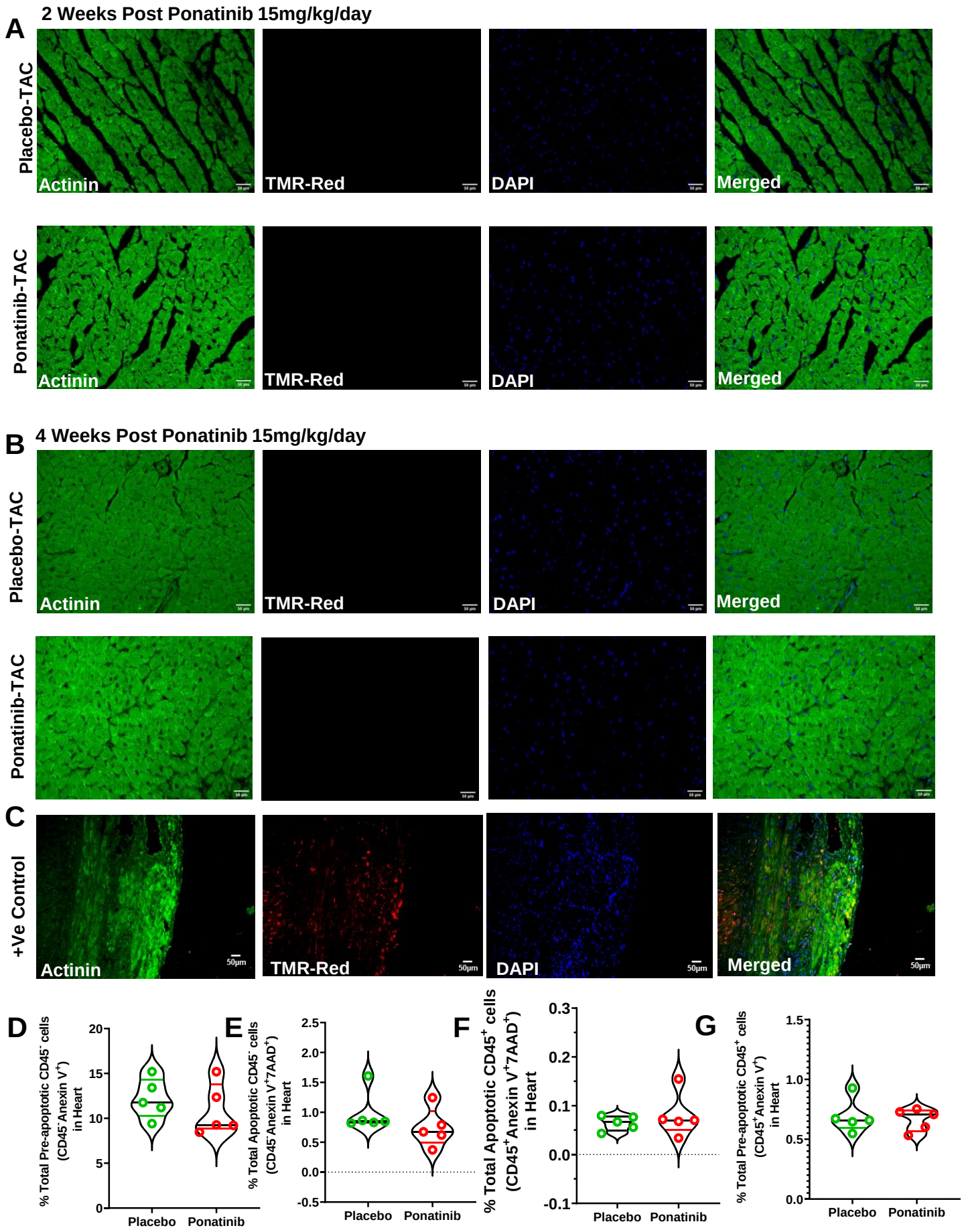

A
2 Weeks Post Ponatinib 15mg/kg/day
Placebo-TAC
Actinin
TMR-Red
DAPI
Merged
Ponatinib-TAC
Actinin
TMR-Red
DAPI
Merged
B
4 Weeks Post Ponatinib 15mg/kg/day
Placebo-TAC
Actinin
TMR-Red
DAPI
Merged
Ponatinib-TAC
C
+Ve Control
Actinin
50µm
TMR-Red
50µm
DAPI
50µm
Merged
50µm
D
% Total Pre-apoptotic CD45- cells (CD45-Anexin V+) in Heart
20
15
10
5
0
Placebo
Ponatinib
E
% Total Apoptotic CD45- cells (CD45-Anexin V+7AAD+) in Heart
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
Placebo
Ponatinib
F
% Total Apoptotic CD45+ cells (CD45+Anexin V+7AAD+) in Heart
0.3
0.2
0.1
0.0
-0.1
Placebo
Ponatinib
G
% Total Pre-apoptotic CD45+ cells (CD45+Anexin V+) in Heart
1.5
1.0
0.5
0.0
Placebo
Ponatinib

Figure S13: Ponatinib treatment unable to induce apoptosis in TAC heart. Eight-week-old C57BL6 wild-type (WT) mice were subjected to TAC surgery. After 1 week, mice were given ponatinib for four weeks (15 mg/Kg/day). Heart sections were obtained from the treated and control groups and subjected to TUNEL assay. **(A-B)** Representative immunofluorescence images of TUNEL stained heart sections. Scale bar = 50 µm. **(C)** Representative immunofluorescence images of +ve TUNEL stained heart sections. The method used to generate positive control: Samples incubated with DNase I (3000U/ml in 50 mM Tris-HCl, pH 7.5, 1mg/ml BSA) for 10 minutes at RT followed by TUNEL assay. **(D-G)** Violin plot represent pre-apoptotic and apoptotic cells in the myocardium of ponatinib-treated TAC mice (4 weeks treated) compared to control. N=5 per group. Data **(D-G)** were analyzed by using the Mann-Whitney U test and represented as mean±SEM.

Figure S14

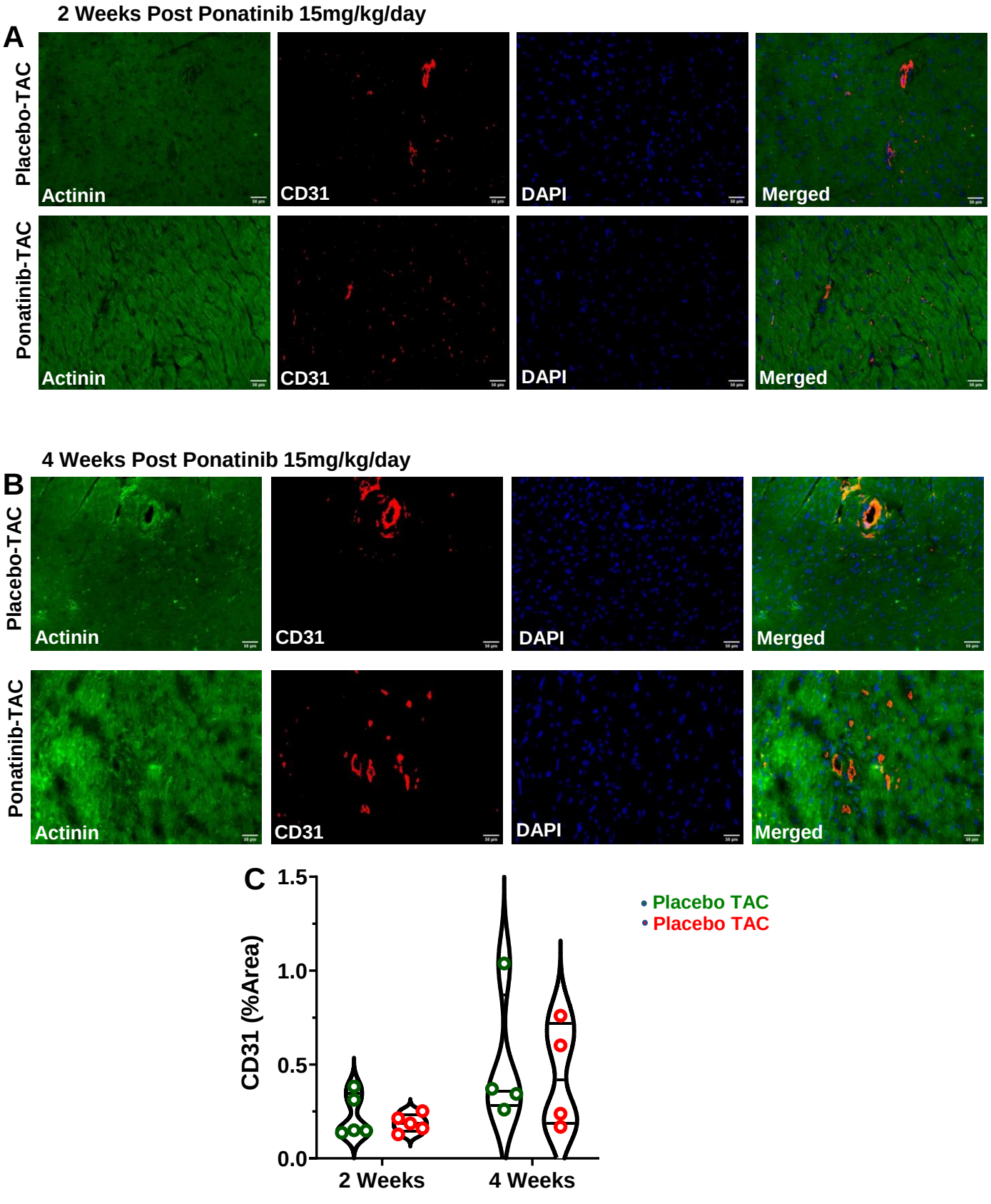
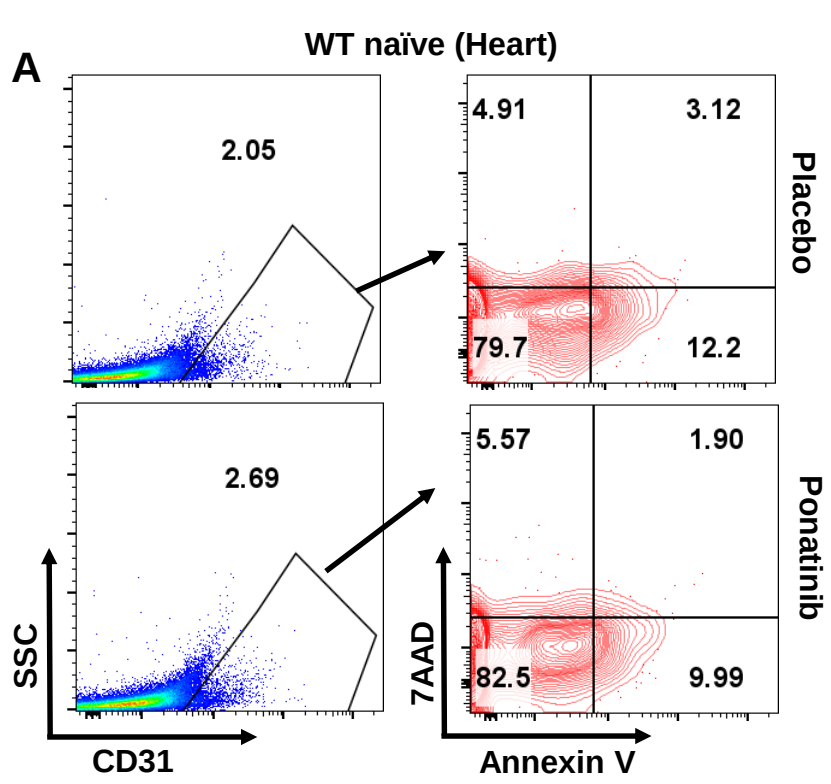
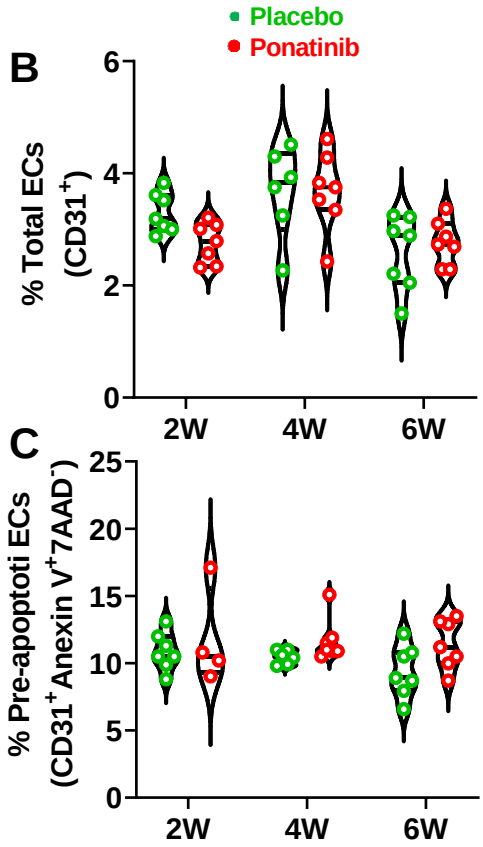
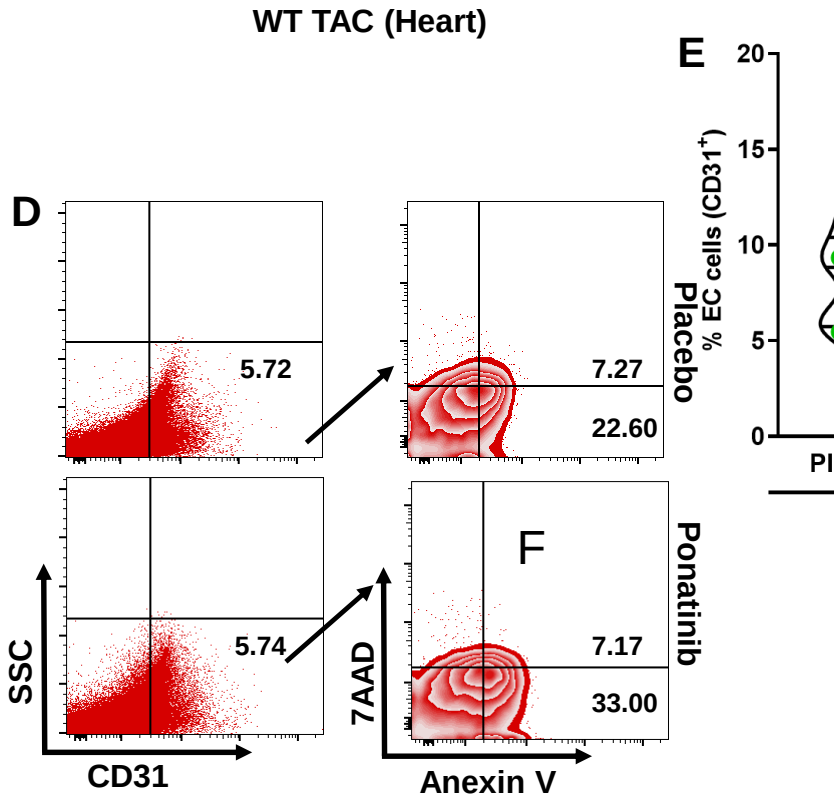

2 Weeks Post Ponatinib 15mg/kg/day
A
Placebo-TAC
Ponatinib-TAC
Actinin
CD31
DAPI
Merged
4 Weeks Post Ponatinib 15mg/kg/day
B
C
CD31 (%Area)
1.5
1.0
0.5
0.0
2 Weeks
4 Weeks
Placebo TAC
Placebo TAC

Figure S14: Ponatinib treatment does not affect vessel densities in the TAC heart. Eight-week-old C57BL6 wild-type (WT) mice were subjected to TAC surgery. After 1 week, mice were given ponatinib for four weeks (15 mg/Kg/day). Heart sections were obtained from the treated and control groups and subjected for CD31. **(A-B)** Representative immunofluorescence images of CD31 stained heart sections. Scale bar = 50 µm. **(C)** Violin plot representing vessel density measurement (as % area) in the myocardium of ponatinib-treated TAC mice as compared to control. At 2 weeks, placebo (N=5), ponatinib (N=5), at 4 weeks, placebo (N=4), ponatinib (N=4). Data were analyzed and significance was compared between Placebo vs Ponatinib for 2 and 4 weeks separately by using the Mann-Whitney U test and represented as mean±SEM

Figure S15


A
WT naïve (Heart)
2.05
4.91
3.12
79.7
12.2
Placebo
2.69
5.57
1.90
82.5
9.99
Ponatinib
SSC
CD31
7AAD
Annexin V


Placebo
Ponatinib
B
% Total ECs (CD31+)
2W
4W
6W
C
% Pre-apoptoti ECs (CD31+Anexin V+7AAD-)
2W
4W
6W


WT TAC (Heart)
D
5.72
7.27
22.60
Placebo
5.74
F
7.17
33.00
Ponatinib
SSC
CD31
7AAD
Anexin V

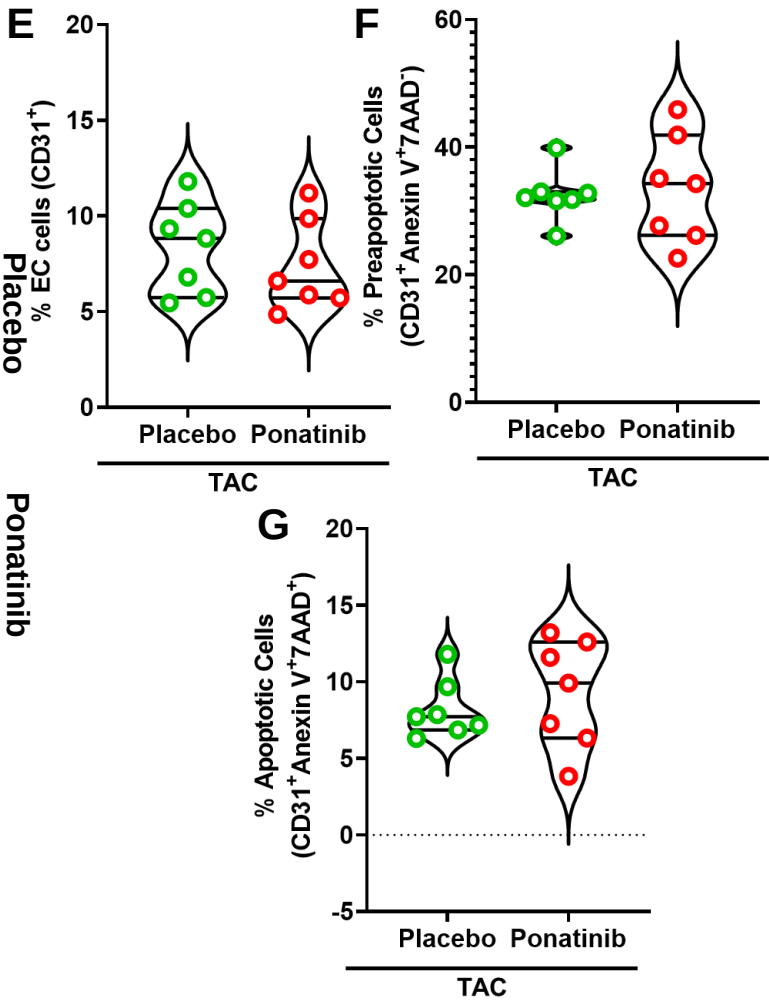

E
% EC cells (CD31+)
Placebo
Ponatinib
TAC
F
% Preapoptotic Cells (CD31+Anexin V+7AAD-)
Placebo
Ponatinib
TAC
G
% Apoptotic Cells (CD31+Anexin V+7AAD+)
Placebo
Ponatinib
TAC

Figure S15: Ponatinib does not affect the endothelial cell population in the treated myocardium. Representative flow cytometry plot and Violin plots showing the total percentage of endothelial cells, pre-apoptotic and apoptotic endothelial cells population in the myocardium of ponatinib treated **(A-C)** naïve mice, **(B)** % total of CD31+ cells, At 2 weeks, placebo (N=7), ponatinib (N=7); At 4 weeks, placebo (N=6), ponatinib (N=7); At 6 weeks, placebo (N=7), ponatinib (N=7). **(C)** % of pre-apoptotic CD31+ cells, At 2 weeks, placebo (N=7), ponatinib (N=4); At 4 weeks, placebo (N=6), ponatinib (N=6); At 6 weeks, placebo (N=7), ponatinib (N=7). Data **(B-C)** were analyzed and significance was compared between Placebo vs Ponatinib for 2, 4 and 6 weeks separately by using the Mann-Whitney U test and represented as mean±SEM **(D-G)** TAC mice(4 weeks ponatinib). **(E)** % of CD31+ cells, placebo (N=7) per group. **(F)** % of pre-apoptotic CD31+ cells (N=7) per group. **(G)** % of apoptotic CD31+ cells (N=7) per group. Data **(E-G)** were analyzed by using the Mann-Whitney U test and represented as mean±SEM.

Figure S16

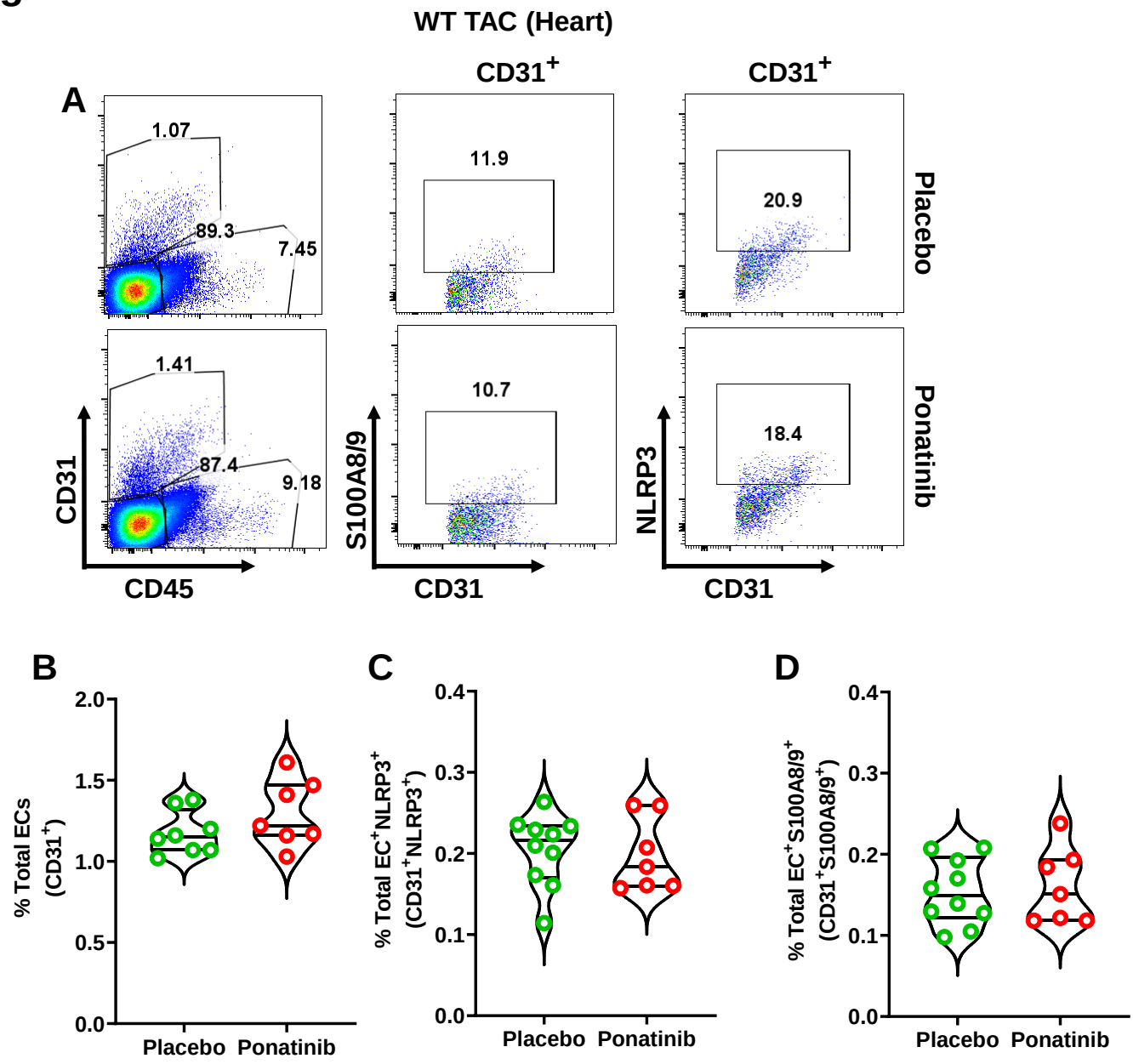


Figure S16: Ponatinib-treated cardiac endothelial cells unable to cause NLRP3 inflammasome activation. The endothelial cell population obtained from ponatinib-treated TAC mice (4 weeks treatment) were stained with S100A8/9 and NLRP3. **(A)** Representative flow cytometry figures show gating strategy to measure endothelial cells from the heart of placebo and ponatinib groups. Data represents quantitation of percent of total **(B)** endothelial cells ($CD31^+$), placebo (N=8), ponatinib (N=7), **(C)** S100A8/9 expressing endothelial cells ($CD31^+S100A8/9^+$), placebo (N=10), ponatinib (N=7), and **(D)** NLRP3 expressing endothelial cells ($CD31^+NLRP3^+$), placebo (N=10), ponatinib (N=7). Data **(B-D)** were analyzed by using the Mann-Whitney U test and represented as mean±SEM.

Figure S17

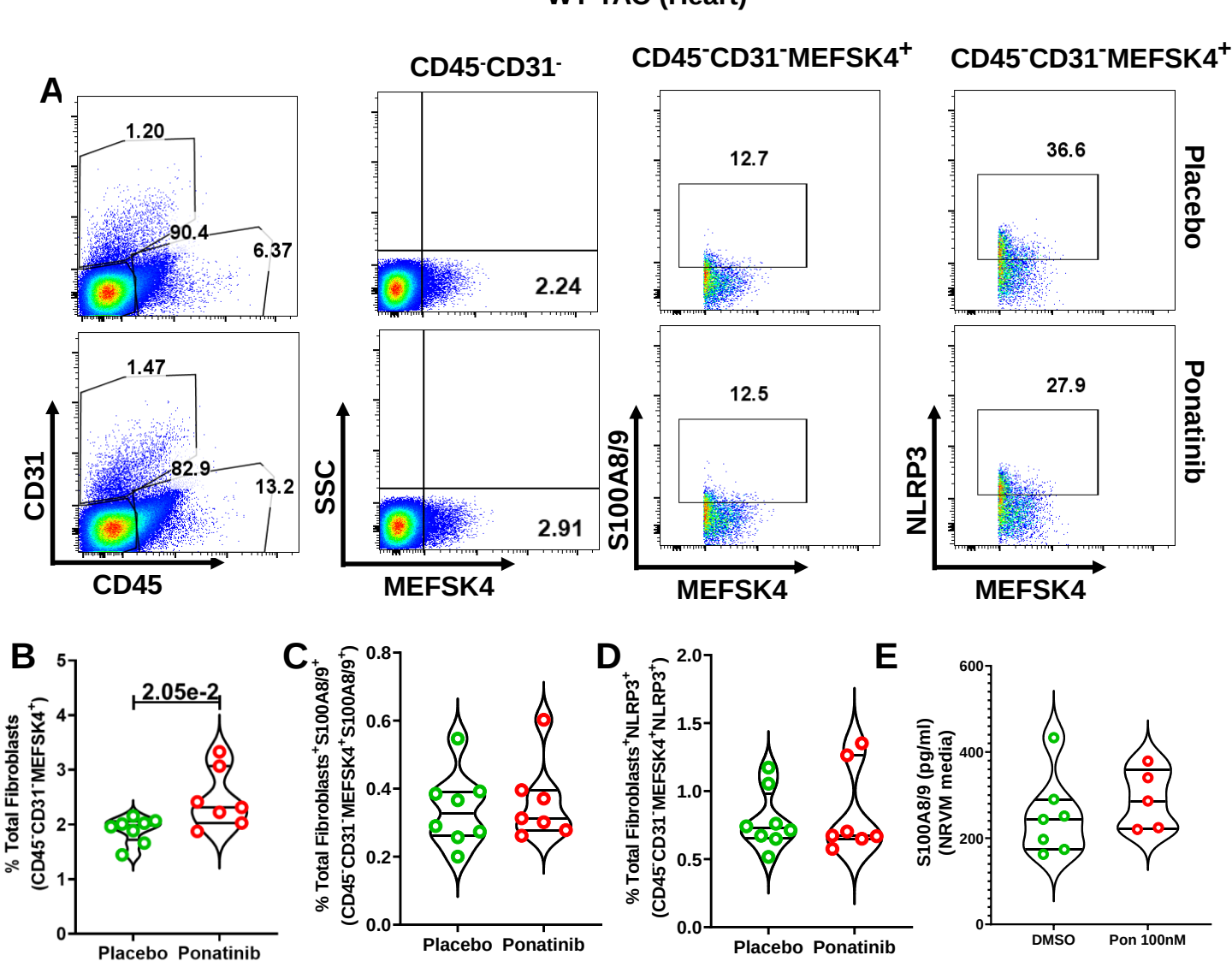


Figure S17: Cardiac fibroblasts were not producing NLRP3 in ponatinib-treated hearts. The fibroblast cell population obtained from ponatinib-treated TAC mice (4 weeks treatment) were stained with S100A8/9 and NLRP3. **(A)** Representative flow cytometry figures show a gating strategy to measure fibroblasts from the heart of placebo and ponatinib groups. Data represents quantitation of percent of total **(B)** fibroblasts ($CD45^-CD31^-MEFSK4^+$), placebo (N=8), ponatinib (N=7), **(C)** S100A8/9 expressing fibroblasts ($CD45^-CD31^-MEFSK4^+S100A8/9^+$), placebo (N=8), ponatinib (N=7), and **(D)** NLRP3 expressing fibroblasts ($CD45^-CD31^-MEFSK4^+NLRP3^+$), placebo (N=8), ponatinib (N=7). **(E)** NRVMs were treated with ponatinib 100nM for 72 hours. Next supernatant was collected, and ELISA was performed to get concentration S100A8/9 in the supernatant, placebo (N=7), ponatinib (N=5). Data **(B-E)** were analyzed by using the Mann-Whitney U test and represented as mean±SEM.

Figure S18

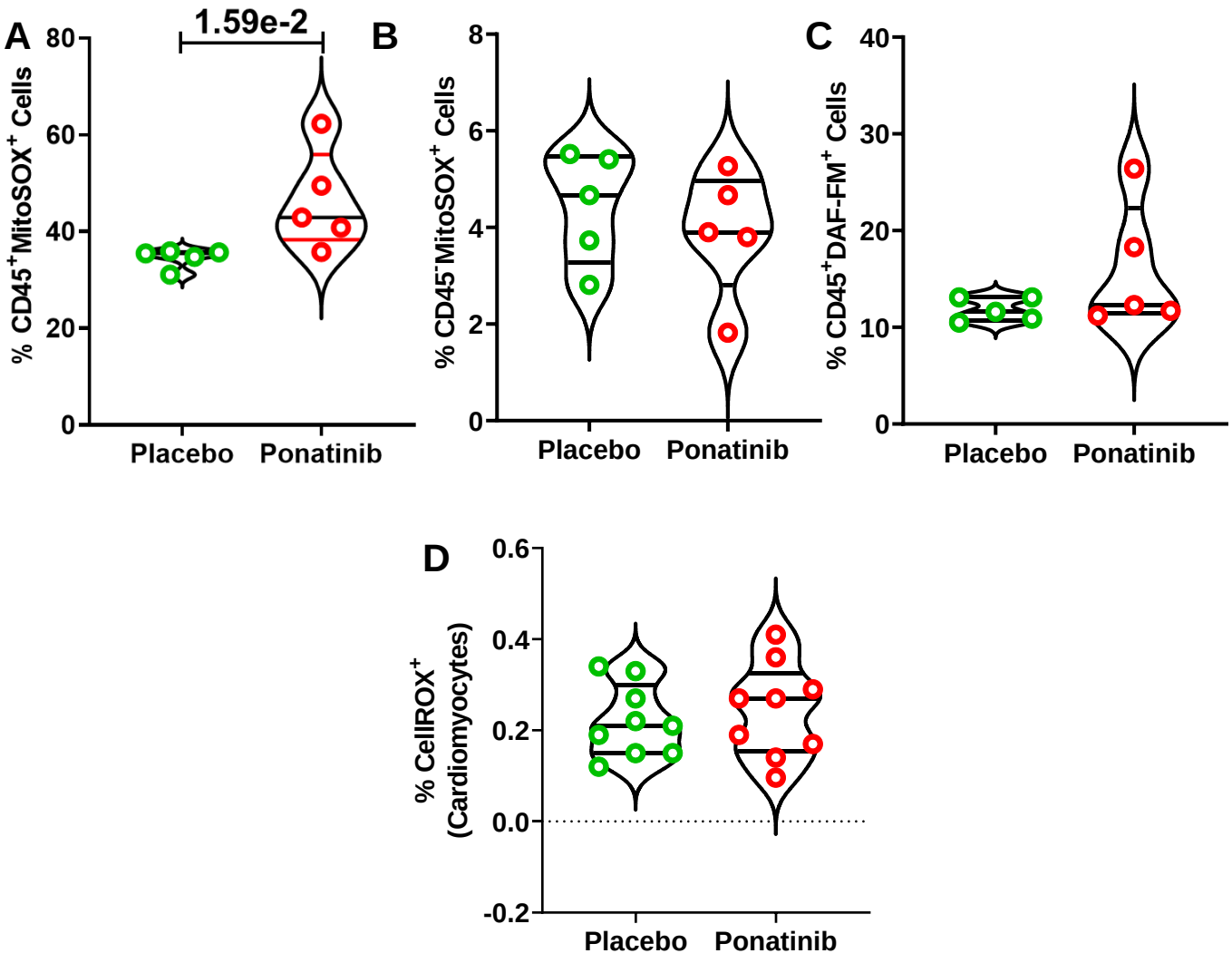


Figure S18: Ponatinib promotes ROS production from CD45⁺ leukocytes but has no effect on non-leukocytes cells. Single-cell suspension was prepared from placebo and ponatinib-treated TAC heart post 4 weeks of treatment. Cells were stained with CD45 antibodies and ROS specific dye MitoSOX, DAF-FM and CellROX, percentage of ROS-producing cells was assessed by flow cytometry. **(A)** percentage of $CD45^{+}MitoSOX^{+}$ cells. **(B)** percentage of $CD45^{-}MitoSOX^{+}$ cells. **(C)** percentage of $CD45^{+}DAF\text{-}FM^{+}$ cells, N=5 per group for (A-C). **(D)** percentage of ROS produced by cardiomyocytes measured with staining of CellROX, N=9 per group. Data **(A-D)** were analyzed by using the Mann-Whitney U test and represented as mean±SEM.

Figure S19

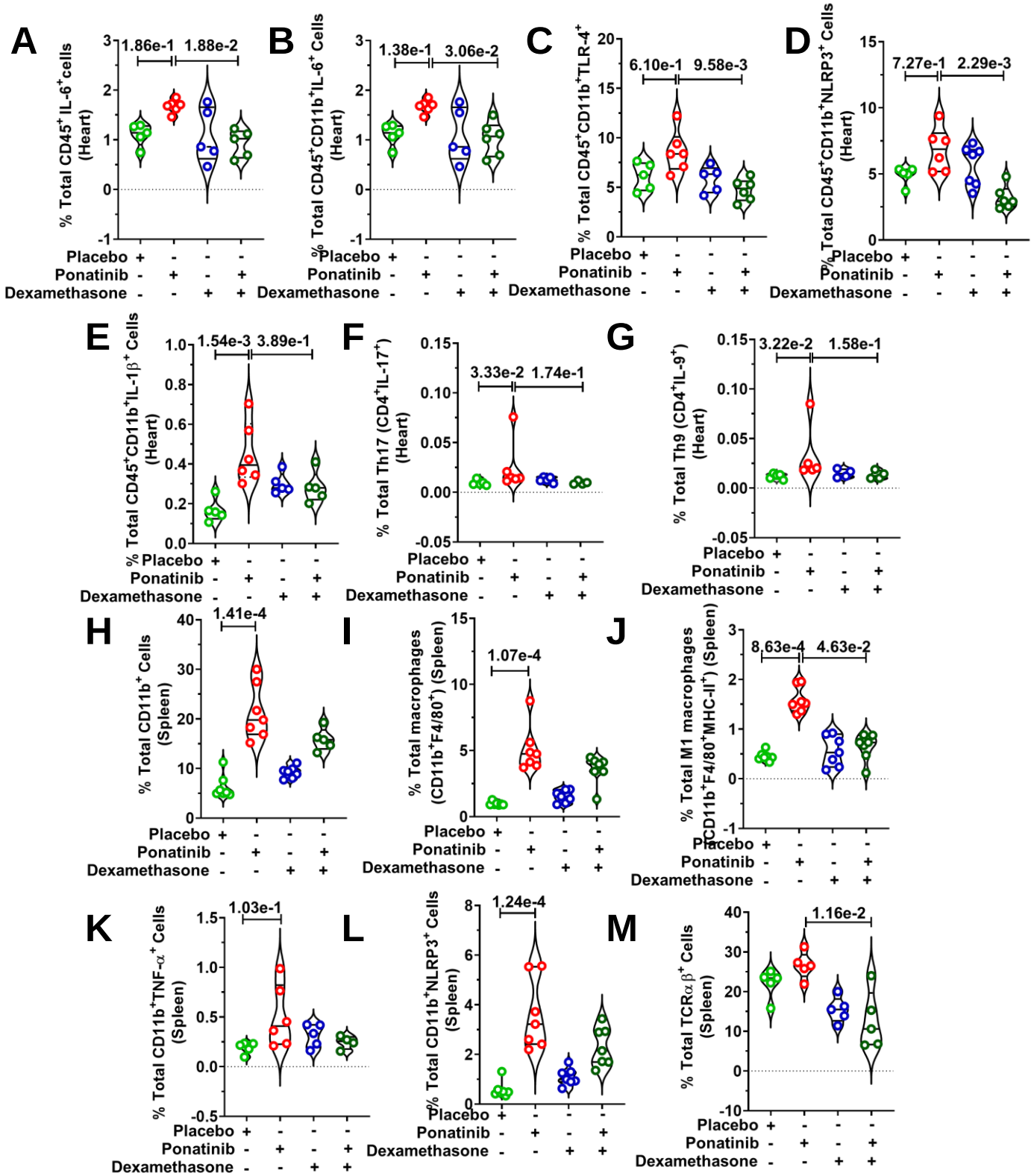

A
% Total CD45+ IL-6+cells (Heart)
1.86e-1
1.88e-2
B
% Total CD45+CD11b+IL-6+ Cells (Heart)
1.38e-1
3.06e-2
C
% Total CD45+CD11b+TLR-4+
6.10e-1
9.58e-3
D
% Total CD45+CD11b+NLRP3+ Cells (Heart)
7.27e-1
2.29e-3
E
% Total CD45+CD11b+IL-1β+ Cells (Heart)
1.54e-3
3.89e-1
F
% Total Th17 (CD4+IL-17+) (Heart)
3.33e-2
1.74e-1
G
% Total Th9 (CD4+IL-9+) (Heart)
3.22e-2
1.58e-1
H
% Total CD11b+ Cells (Spleen)
1.41e-4
I
% Total macrophages (CD11b+F4/80+) (Spleen)
1.07e-4
J
% Total M1 macrophages (CD11b+F4/80+MHC-II+) (Spleen)
8.63e-4
4.63e-2
K
% Total CD11b+TNF-α+ Cells (Spleen)
1.03e-1
L
% Total CD11b+NLRP3+ Cells (Spleen)
1.24e-4
M
% Total TCRαβ+ Cells (Spleen)
1.16e-2
Placebo
Ponatinib
Dexamethasone

Figure S19: Dexamethasone rescued ponatinib-induced cardiotoxicities by suppressing excessive inflammation. Eight-week-old C57BL6 wild-type (WT) mice were subjected to TAC surgery followed by ponatinib treatment (15 mg/Kg/day) after 1 week. Mice were given dexamethasone (2 mg/Kg i.p.) three times a week and concurrently ponatinib for four weeks. Only TAC groups have been taken for this study. **(A-M)** Quantitation of immune cells as a percentage of total cells isolated from heart of animal groups mentioned in the figures. Data represents quantitation of percent of total **(A)** IL-6 producing leukocytes ($CD45^+IL\text{-}6^+$), placebo (N=5), ponatinib (N=6), dexamethasone (N=5) and ponatinib + dexamethasone (N=5), **(B)** IL-6 producing myeloid cells ($CD45^+CD11b^+IL\text{-}6^+$), placebo (N=5), ponatinib (N=6), dexamethasone (N=5) and ponatinib + dexamethasone (N=6), **(C)** TLR-4 expressing myeloid cells ($CD45^+CD11b^+TLR\text{-}4^+$), placebo (N=5), ponatinib (N=6), dexamethasone (N=5) and ponatinib + dexamethasone (N=6), **(D)** NLRP3+monocytes ($CD45^+CD11b^+NLRP3^+$), placebo (N=5), ponatinib (N=6), dexamethasone (N=7) and ponatinib + dexamethasone (N=6), **(E)** IL-1β producing myeloid cells ($CD45^+CD11b^+IL\text{-}1\beta^+$), placebo (N=5), ponatinib (N=6), dexamethasone (N=5) and ponatinib + dexamethasone (N=5), **(F)** Th17 cells ($CD4^+IL\text{-}17^+$), placebo (N=5), ponatinib (N=5), dexamethasone (N=5) and ponatinib + dexamethasone (N=4), and **(G)** Th9 cells ($CD4^+IL\text{-}9^+$), placebo (N=5), ponatinib (N=5), dexamethasone (N=4) and ponatinib + dexamethasone (N=4). **(H-M)** Quantitation of immune cells as a percentage of total cells isolated from spleen of animal groups mentioned in the figures. Data represents quantitation of percent of total **(H)** myeloid cells ($CD11b^+$), placebo (N=7), ponatinib (N=7), dexamethasone (N7) and ponatinib + dexamethasone (N=5), **(I)** macrophages ($CD11b^+F4/80^+$), placebo (N=7), ponatinib (N=7), dexamethasone (N=7) and ponatinib + dexamethasone (N=7), **(J)** M1 macrophages ($CD11b^+F4/80^+MHC\text{-}II^+$), placebo (N=7), ponatinib (N=7), dexamethasone (N=7) and ponatinib + dexamethasone (N=7), **(K)** TNF-α producing myeloid cells ($CD11b^+TNF\text{-}\alpha^+$), placebo (N=5), ponatinib (N=6), dexamethasone (N=5) and ponatinib + dexamethasone (N=4), **(L)** $NLRP3^+$ myeloid cells ($CD11b^+$), placebo (N=7), ponatinib (N=7), dexamethasone (N=7) and ponatinib + dexamethasone (N=7), and **(M)** T cells ($TCR\alpha\beta^+$), placebo (N=5), ponatinib (N=5), dexamethasone (N=5) and ponatinib + dexamethasone (N=5). Data **(A-M)** were analyzed by using Kruskal-Wallis followed by Dunn test and represented as mean±SEM.

Figure S20

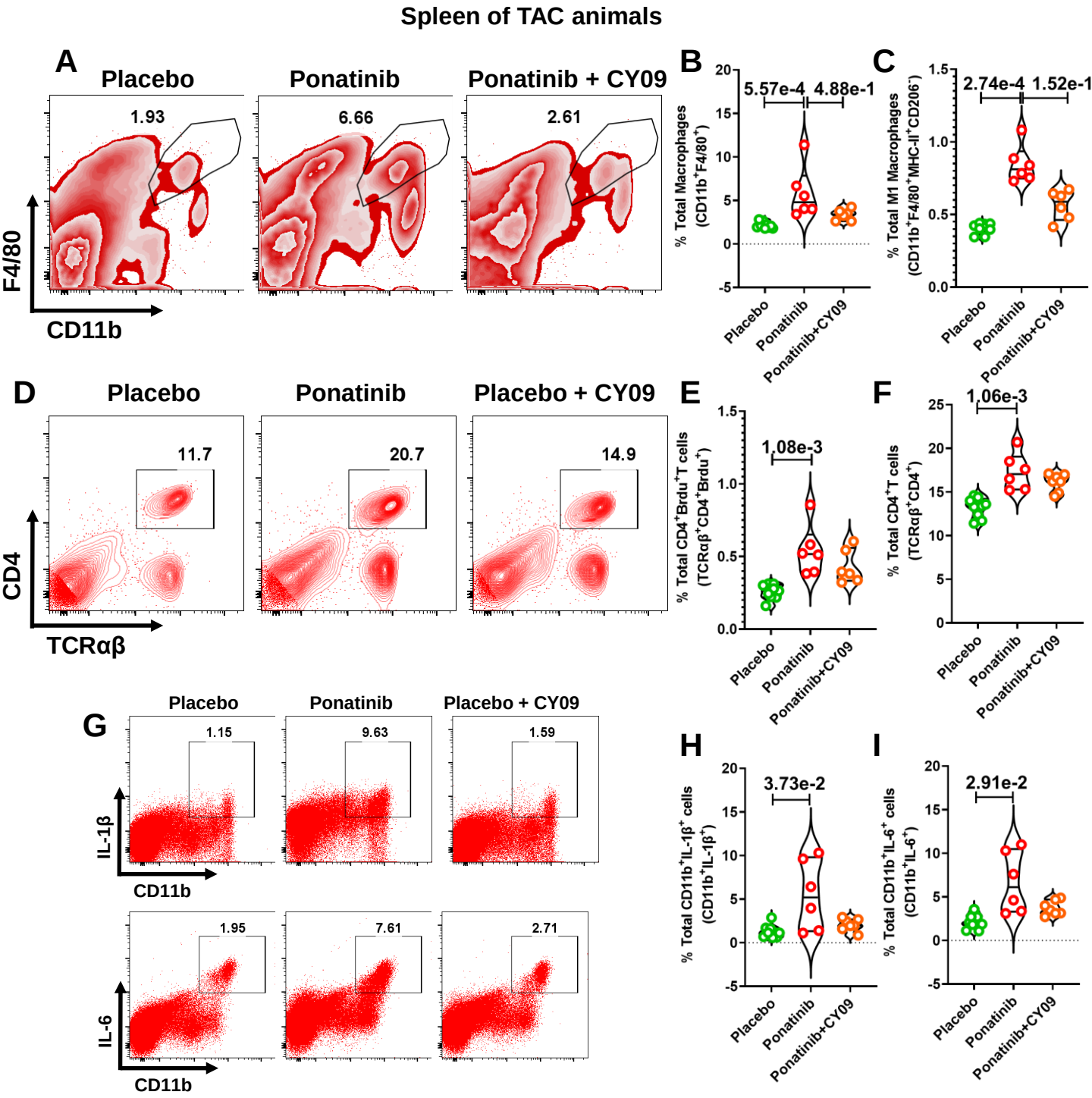

Spleen of TAC animals
A
Placebo
Ponatinib
Ponatinib + CY09
1.93
6.66
2.61
F4/80
CD11b
B
5.57e-4
4.88e-1
% Total Macrophages (CD11b+F4/80+)
C
2.74e-4
1.52e-1
% Total M1 Macrophages (CD11b+F4/80+MHC-II+CD206-)
Placebo
Ponatinib
Ponatinib+CY09
D
Placebo
Ponatinib
Placebo + CY09
11.7
20.7
14.9
CD4
TCRαβ
E
1.08e-3
% Total CD4+Brdu+T cells (TCRαβ+CD4+Brdu+)
F
1.06e-3
% Total CD4+T cells (TCRαβ+CD4+)
G
Placebo
Ponatinib
Placebo + CY09
1.15
9.63
1.59
IL-1β
CD11b
1.95
7.61
2.71
IL-6
CD11b
H
3.73e-2
% Total CD11b+IL-1β+ cells (CD11b+IL-1β+)
I
2.91e-2
% Total CD11b+IL-6+ cells (CD11b+IL-6+)

Figure S20: CY-09 ameliorates ponatinib-induced immune activation in spleen. Eight-week-old C57BL6 wild-type (WT) mice were subjected to TAC surgery. After 1 week, mice were given CY-09 (2 mg/Kg i.p.) six times a week and concurrently ponatinib for four weeks (15 mg/Kg/day). Only TAC groups have been taken for this study. Spleens were taken from these mice and macerated to obtain a single-cell suspension. Next, cells were stained, and flow cytometry was performed. **(A)** Representative figure of flow cytometry showing gating strategy to measure macrophage populations. Data represents quantitation of percent of total **(B)** macrophages ($CD11b^+F4/80^+$), placebo (N=9), ponatinib (N=6), and ponatinib + CY09 (N=6), and **(C)** M1 macrophages ($CD11b^+F4/80^+MHC\text{-}II^+CD206^-$), placebo (N=8), ponatinib (N=6), and ponatinib + CY09 (N=6). **(D)** Representative figure of flow cytometry showing gating strategy to measure T cells. Data represents the quantitation of percent of the total **(E)** proliferating CD4 T cells ($TCR\alpha\beta^+CD4^+Brdu^+$), placebo (N=9), ponatinib (N=6), and ponatinib + CY09 (N=6), and **(F)** total CD4 T cells ($TCR\alpha\beta^+CD4^+$), placebo (N=9), ponatinib (N=6), and ponatinib + CY09 (N=7). **(G)** Representative figure of flow cytometry showing gating strategy to measure myeloid cells expressing IL-1β and IL-6. Data represents quantitation of percent of total **(H)** IL-1β producing cells ($CD11b^+IL\text{-}1\beta^+$), placebo (N=9), ponatinib (N=6), and ponatinib + CY09 (N=7), and **(I)** IL-6 producing cells ($CD11b^+IL\text{-}6^+$), placebo (N=10), ponatinib (N=6), and ponatinib + CY09 (N=7). Data **(B,C,E,F and H, I)** were analyzed by using Kruskal-Wallis followed by Dunn test and represented as mean±SEM.

Figure S21

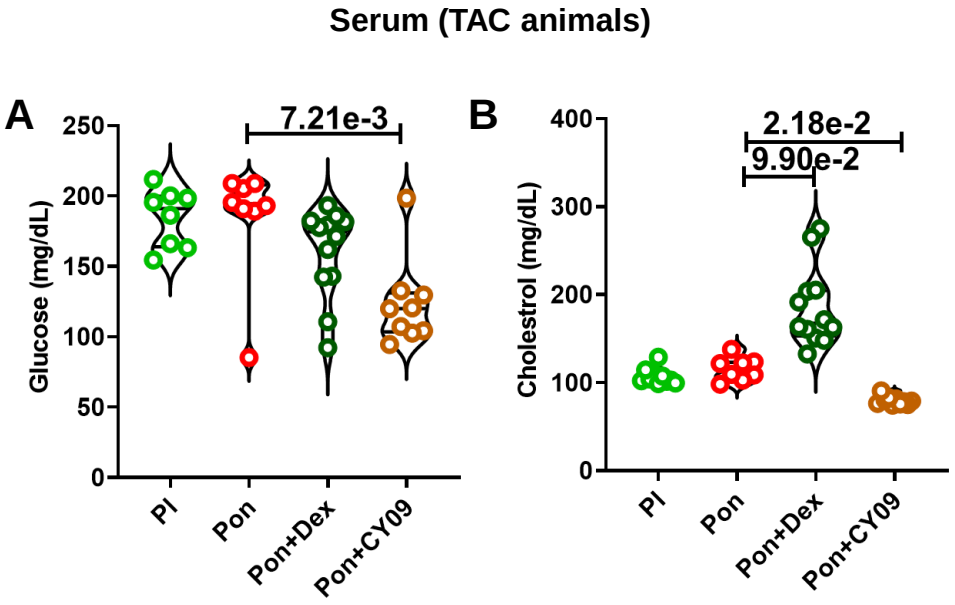


Figure S21: CY-09 regulates Glucose and Cholesterol levels in circulation. (A) Blood glucose, placebo (N=8), ponatinib (N=8), ponatinib + dexamethasone (N=12) and ponatinib + CY09 (N=9), and **(B)** Blood cholesterol concentrations placebo (N=10), ponatinib (N=8), ponatinib + dexamethasone (N=12) and ponatinib + CY09 (N=10). Data **(A,B)** were analyzed by using Kruskal-Wallis followed by Dunn test and represented as mean±SEM.

Figure S22

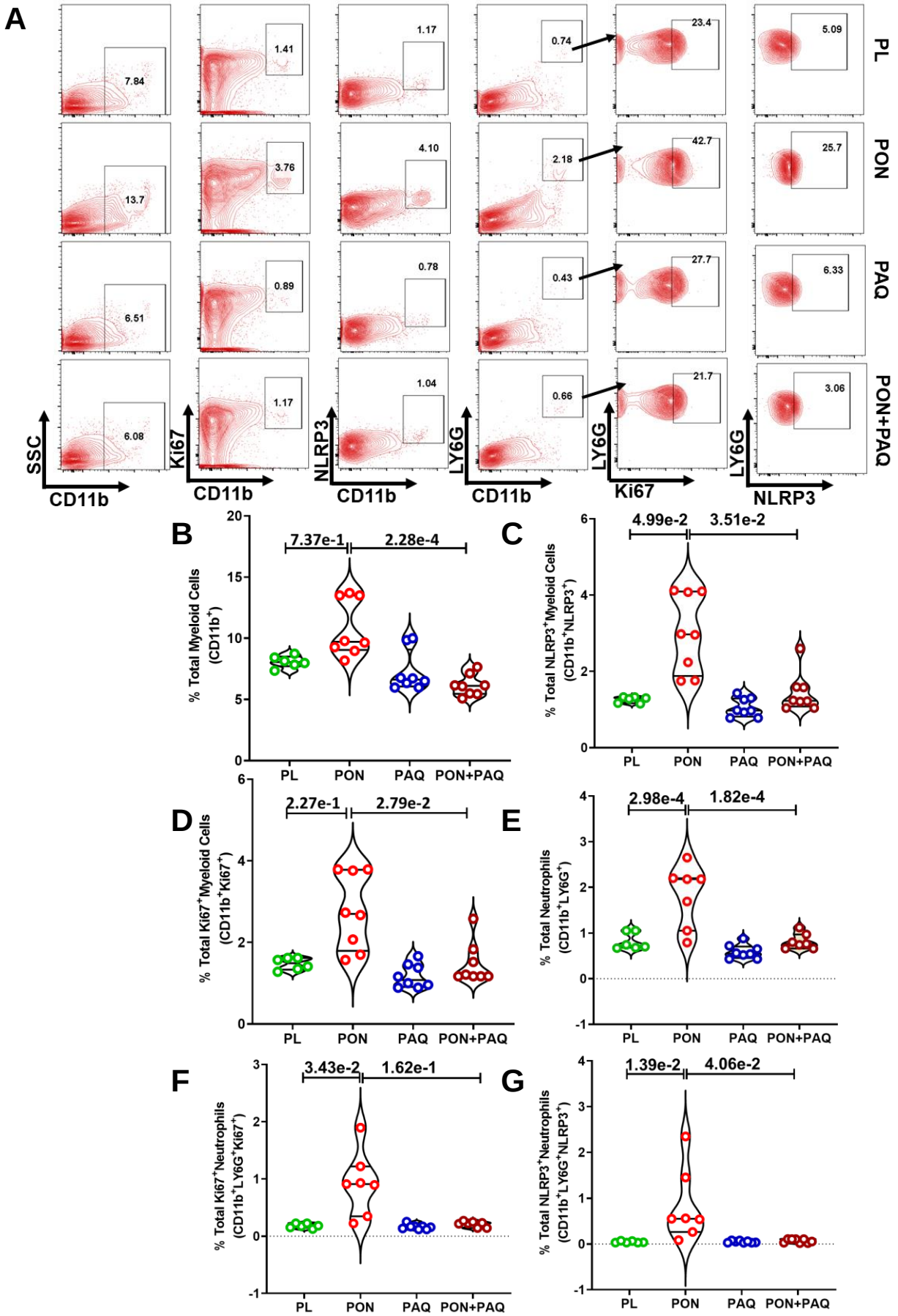

A
PL
PON
PAQ
PON+PAQ
SSC
CD11b
Ki67
CD11b
NLRP3
CD11b
LY6G
CD11b
LY6G
Ki67
LY6G
NLRP3
B
% Total Myeloid Cells (CD11b+)
7.37e-1
2.28e-4
C
% Total NLRP3+Myeloid Cells (CD11b+NLRP3+)
4.99e-2
3.51e-2
D
% Total Ki67+Myeloid Cells (CD11b+Ki67+)
2.27e-1
2.79e-2
E
% Total Neutrophils (CD11b+LY6G+)
2.98e-4
1.82e-4
F
% Total Ki67+Neutrophils (CD11b+LY6G+Ki67+)
3.43e-2
1.62e-1
G
% Total NLRP3+Neutrophils (CD11b+LY6G+NLRP3+)
1.39e-2
4.06e-2

Figure S22: Paquinimod ameliorates ponatinib-induced immune activation in the spleen. Eight-week-old C57BL6 wild-type (WT) mice were subjected to TAC surgery. After 1 week, mice were given paquinimod (3.75 mg/Kg ad libitum in drinking water daily) and concurrently ponatinib for four weeks (15 mg/Kg/day). Only TAC groups have been taken for this study. Spleens were taken from these mice and macerated to obtain a single-cell suspension. Next, cells were stained, and flow cytometry was performed. **(A)** Representative figure of flow cytometry showing gating strategy to measure immune cell populations. Data represents quantitation of percent of total **(B)** total myeloid cells ($CD11b^+$), placebo (N=6), ponatinib (N=8), paquinimod (N=8) and ponatinib + paquinimod (N=8), **(C)** total NLRP3 expressing myeloid cells ($CD11b^+NLRP3^+$), placebo (N=6), ponatinib (N=8), paquinimod (N=8) and ponatinib + paquinimod (N=8), **(D)** total proliferating myeloid cells ($CD11b^+Ki67^+$), placebo (N=6), ponatinib (N=8), paquinimod (N=8) and ponatinib + paquinimod (N=8), **(E)** total neutrophils ($CD11b^+LY6G^+$), placebo (N=6), ponatinib (N=7), paquinimod (N=8) and ponatinib + paquinimod (N=7), **(F)** total proliferating neutrophils ($CD11b^+LY6G^+Ki67^+$), placebo (N=6), ponatinib (N=6), paquinimod (N=8) and ponatinib + paquinimod (N=7), and **(G)** total NLRP3 expressing neutrophils ($CD11b^+LY6G^+NLRP3^+$), placebo (N=6), ponatinib (N=7), paquinimod (N=8) and ponatinib + paquinimod (N=8). Data **(B-G)** were analyzed by using Kruskal-Wallis followed by Dunn test and represented as mean±SEM.

Figure S23

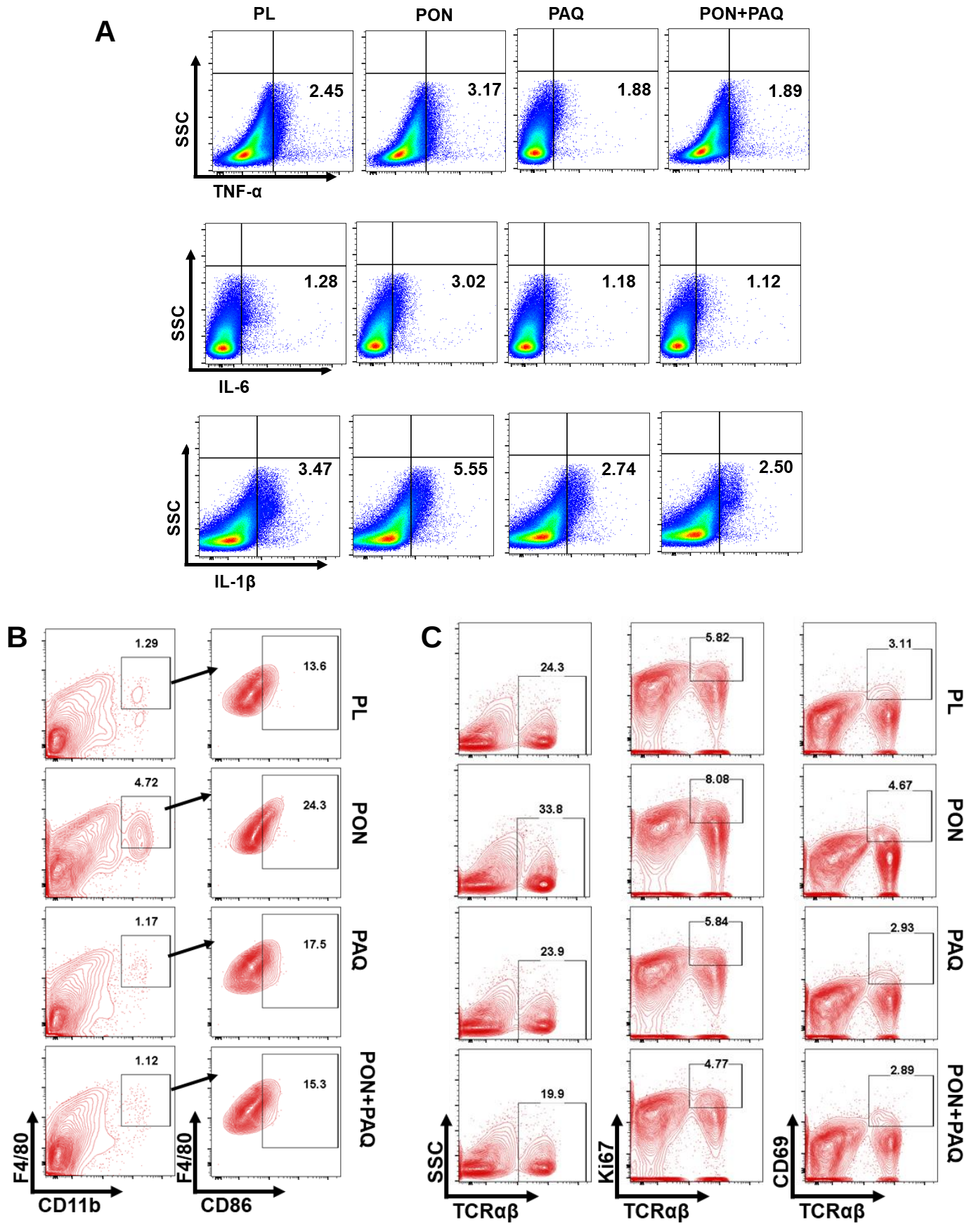

A
PL
PON
PAQ
PON+PAQ
SSC
TNF-α
2.45
3.17
1.88
1.89
IL-6
1.28
3.02
1.18
1.12
IL-1β
3.47
5.55
2.74
2.50
B
1.29
13.6
4.72
24.3
1.17
17.5
1.12
15.3
F4/80
CD11b
CD86
C
24.3
5.82
3.11
33.8
8.08
4.67
23.9
5.84
2.93
19.9
4.77
2.89
TCRαβ
Ki67
CD69

Figure S23: Paquinomod ameliorates ponatinib-induced immune activation in the spleen. Eight-week-old C57BL6 wild-type (WT) mice were subjected to TAC surgery. After 1 week, mice were given paquinimod and concurrently ponatinib for four weeks (15 mg/Kg/day). Only TAC groups have been taken for this study. Spleens were taken from these mice and macerated to obtain a single-cell suspension. Next, cells were stained, and flow cytometry was performed. **(A-C)** A representative flow cytometry figure shows gating strategy to measure cytokine production, M1 macrophages, and T cell populations.

Figure S24

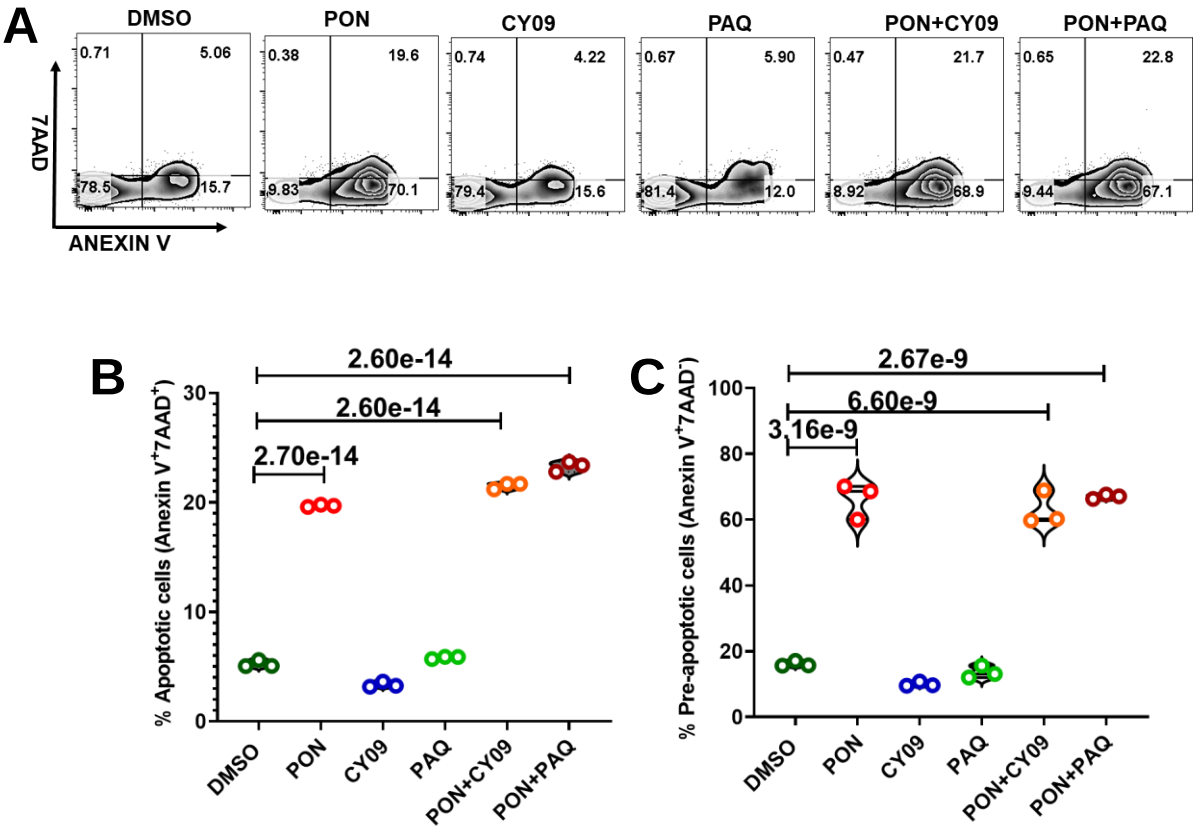


Figure S24: CY09 or paquinimod do not interfere ponatinib efficacy *in-vitro*. (A) Flow cytometry plots representing Annexin V and 7AAD positive K562 cell populations in ponatinib and inhibitor-treated groups. **(B-C)** total apoptotic and pre-apoptotic K562 cells after ponatinib and inhibitor treatment. Experiment was repeated three times (N= 3). Data **(B-C)** were analyzed by using ordinary one way ANOVA followed by Tukey's multiple comparisions test and represented as mean±SEM.