Chronic sympathetic driven hypertension promotes atherosclerosis by enhancing hematopoiesis

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Supplementary file

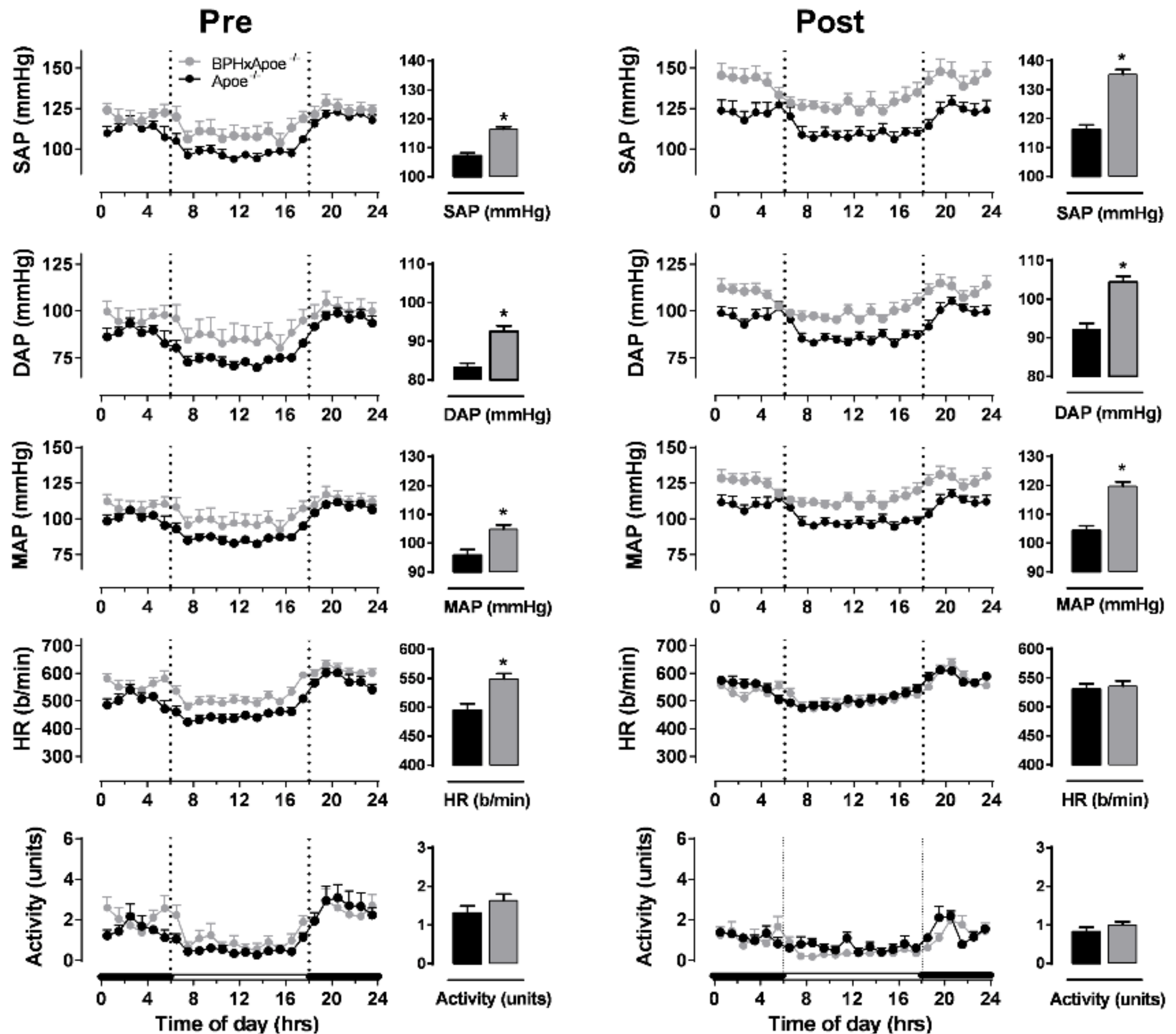

Figure S1. Cardiovascular characteristics. *Apoe*$^{-/-}$ and *BPH/Apoe*$^{-/-}$ mice were fed a WTD for 16 wks. Using radiotelemetry mice were assessed for SAP, DAP, MAP along with HR and activity. These measurements were recorded pre and post-WTD. Data are presented as mean ± SEM where *$p<0.05$ (Student's t-test) n=10

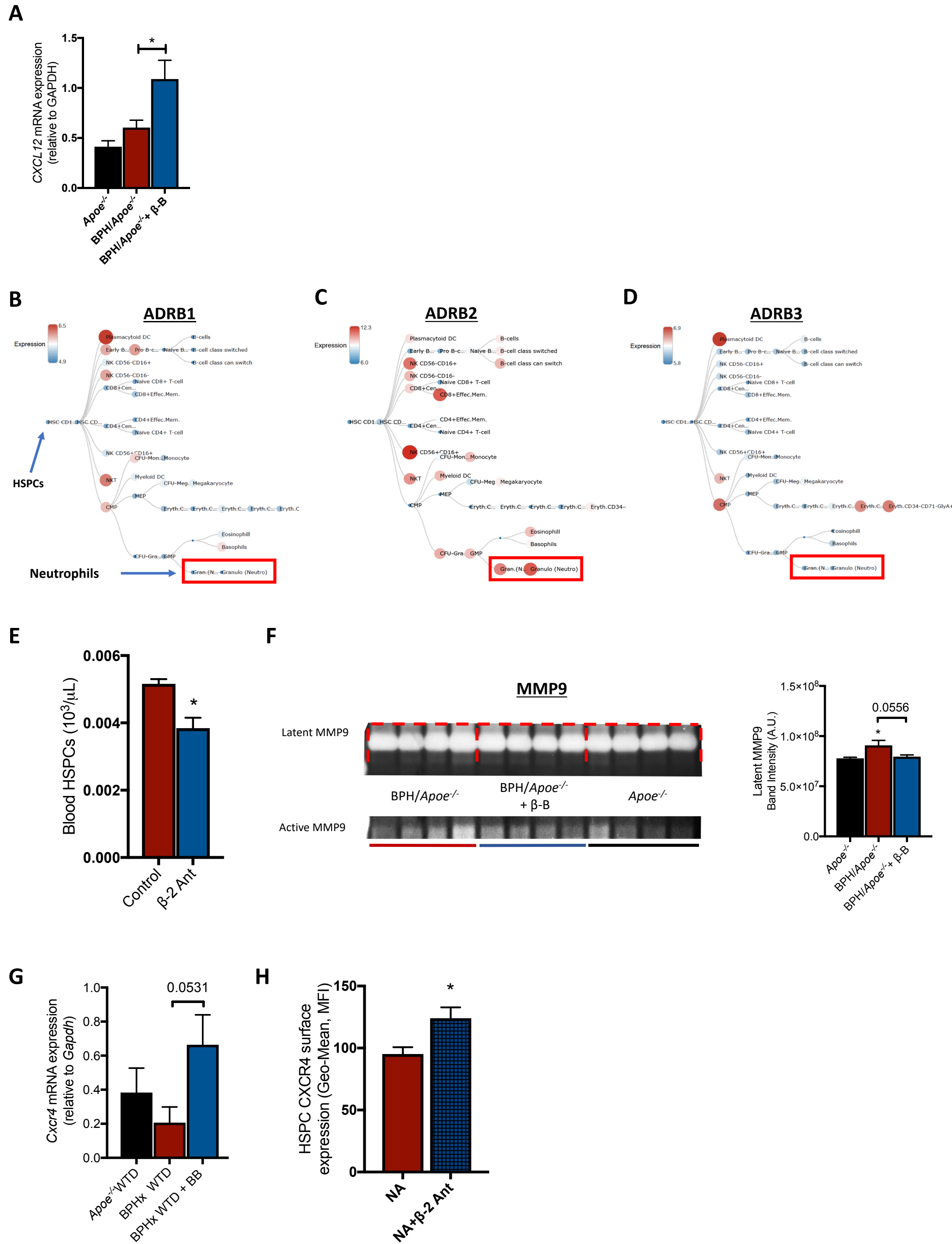

Figure S2. Bone marrow microenvironment alteration in sympathetic activation. A) Real time qPCR was used to assess mRNA expression levels of BM *Cxcl12*. **B-D)** BloodSpot (Online software) generation of a hierarchal myeloid tree depicting the expression of the adrenergic receptors β-1, β-2, β-3. **E)** *BPH/Apoe*$^{+/+}$ mice were treated with the β_2 specific antagonist ICI-118551 for 2 weeks and blood HSPCs assessed via flow cytometry. **F)** Latent MMP9 levels assessed by gelatin zymography. **G)** mRNA expression levels of BM *Cxcr4*. **H)** BM neutrophils were stimulated with NA ± ICI-118551 and the harvested supernatant was incubated with BM HSPCs to assess CXCR4 cleavage via flow cytometry**.** Data are presented as mean ± SEM where *p<0.05 measured by a One-Way ANOVA. **A)** n=5-9**, E)** n=4, **F)** n= 4, **G)** n=5-9, **H)** n=6.

Antibodies	Cat #	Clone	Source	Fluorochrome
CD45	103126	30-F11	BioLegend	PB
Gr1	552093	RB6-8C5	BD Pharmingen	PerCP-Cy5.5
CD115	12-1152-82	AFS98	eBioscience	PE
Gr1	553127	RB6-8C5	BD Bioscience	FITC
CD2	11-0021-85	RM2-5	eBioscience	FITC
CD3	11-0033-82	eBio500A2	eBioscience	FITC
CD19	101506	MP19-1	BioLegend	FITC
TER119	11-5921-85	TER-119	eBioscience	FITC
CD45R	11-0452-85	RA3-6B2	eBioscience	FITC
CD8a	553030	53-6.7	BD Bioscience	FITC
CD4	11-0042-85	RM4-5	eBioscience	FITC/PE-Cy7
Sca1	108120	D7	BioLegend	PB
ckit	105826	2B8	BioLegend	APC/CY7
Fcγ	560540	2.4G2	BD Biosciences	Percp-Cy5.5
CD31	46-0311-82	390	eBioscience	Percp-Cy5.5
CD51	12-0512-83	RMV-7	eBioscience	PE
F4/80	123114	BM8	BioLegend	PE-Cy7
CXCR4	12-9991-82	2B11	eBioscience	PE

Table S1. Antibody details

Gene	Primer Sequence – forward	Primer sequence – Reverse
GAPDH	5' – TGA AGC AGG CAT CTG AGG G	5' – CGA AGG TGG AAG AGT GGG AG
CXCL12	5' – CGC CAA GGT CGT CGC CG	5' – TTG GCT CTG GCG ATG TGG C
CXCR4	5' – CAC GGC TGT AGA GCG AGT GT	5' – TGC CGA CTA TGC CAG TCA AG
Runx2	5' – TCC GAA ATG CCT CCG CTG TTA T	5' – GGA CCG TCC ACT GTC ACT TTA A

Table S2. qRT-PCR primer sequences

Methods

Animal Models: *Apoe*$^{-/-}$ mice were purchased from Jackson Laboratories and bred at the AMREP Animal centre. To generate hypertensive *Apoe*$^{-/-}$ mice, BPH/2J mice were crossed with *Apoe*$^{-/-}$ mice to produce BPH/2J x *Apoe*$^{-/-}$ (BPH/*Apoe*$^{-/-}$) mice. At 6 weeks of age, male *Apoe*$^{-/-}$ and BPH/ *Apoe*$^{-/-}$ mice were placed on a western type diet (WTD - SF00-219, Specialty Feeds, Australia. 21% fat, 0.15% cholesterol) for 16 weeks. In the first cohort of mice, age-matched mice *Apoe*$^{-/-}$ and BPH/*Apoe*$^{-/-}$ were placed on a WTD for 16 weeks for end-point analysis. In a second cohort of mice, obtained from a new set of breeders, three groups of aged-matched mice were employed: 1) *Apoe*$^{-/-}$, 2) BPH/*Apoe*$^{-/-}$and 3) BPH/ *Apoe*$^{-/-}$ + propranolol (0.5g/L; administered via drinking water for the duration of the WTD feeding). For the propranolol group, mice consumed on average 2.5ml of water amounting to an average daily dose of 35-40mg/kg/daily of propranolol.

To determine the effect of specific β_2-adrenoreceptor blockade on HSPC mobilization and blood pressure we used BPH mice on an *Apoe*$^{+/+}$ background. The mice were injected daily with ICI-118551 (5mg/kg; Abcam, AUS) for 2 weeks.

All animal experiments were approved by the AMREP Animal Ethics Committee and conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes as stipulated by the National Health and Medical Research Council of Australia. All mice were housed in a normal light and dark cycle and had ad libitum access to food and water.

Lesion Analysis: Mice were perfused with saline before heart and aorta collection. The aorta was dissociated from the heart at the point of entry between the highest point of the atria and fixed in PFA for further analysis (described below). The heart was dissected so that the upper region of the heart containing the aortic sinus was retained and frozen in optimal cutting temperature (OCT) compound. Serial 6µm sections of the proximal aorta were harvested on a cryostat at -20°C. Images were captured on an Olympus BX61 microscope, whereas collagen was imaged using polarised light. Image quantification was performed using Adobe Photoshop CC.

H&E staining: Sections were fixed (4min, 10% neutral buffered formalin), washed in PBS (4min), stained in Mayer's Haematoxylin (15min) and washed with running tap water before blueing in Scott's tap water for 30secs. The slides were then put in 95% ethanol (10 dips), stained in buffered alcoholic eosin (8min), dehydrated in absolute ethanol, cleared with xylene and coverslips were mounted using depex.

Oil Red O (lipid) staining: Sectioned lesions were fixed in 10% buffered formalin (4mins), washed in PBS (4min), dipped in 60% isopropanol before staining in 60% ORO working solution (2hrs, stock solution: 1% ORO powder in isopropanol). The slides were then washed in 60% isopropanol and distilled water. Sections were stained in Mayer's Haematoxylin (4mins), washed in tap and distilled water (3min each) and mounted with aquamount.

CD68 (macrophage) staining: Sections were thawed and fixed with paraformaldehyde (4%, 20min), washed in PBS (2x5min), incubated in pre-chilled 3% H_2O_2 in methanol (20min) and then washed in PBS (2x5min). Each section was blocked with normal goat serum (NGS, 10%,

30min), incubated with AVIDIN blocking solution (15min), rinsed in PBS and then incubated with rat anti-mouse CD68 primary antibody (1:200, 5% NGS, 4°C) overnight. The slides were then washed in PBS (2x5min) before being incubated with the secondary antibody (1:100, 5% NGS, 30min). Next, the sections were washed in PBS (2x5min), incubated with ABC avidin/biotin complex (30min) and DAB solution. Staining reaction was terminated with distilled water. The sections were counterstained with Mayer Haematoxylin for 15sec and rinsed in tap water before blueing in scotts tap water and washing in tap water. Finally, slides were dehydrated in ethanol (95% 3min, 100% 3x3min), cleared in xylene (2x5min) and mounted with depex.

Picosirius (collagen) staining: Sections were thawed and fixed in pre-chilled acetone (15min), washed in PBS (2x5min), stained in 0.1% Sirius red F3BA (1hr) and then washed in 0.01M HCl (2min). Subsequently, the slides were then dehydrated in alcohol (95%, 5mins; 100%, 2x5min), cleared in xylene (2x5min) and mounted with depex. Sections were imaged on Olympus BX61 microscope under brightfield and polarised light.

Aortic Arch Lipid Analysis: Lipid content, as a readout of atherosclerotic plaques in the aortic arch was measured by *en face* analysis. Dissected aortas were fixed in PFA, with fat and connective tissue removed from the outer layers of the vessel prior to staining. The aorta was then cut longitudinally and stained with ORO. After washing, the stained aortas were mounting on a silicone coated dish. Aortas were viewed on an Olympus SZX10 and captured using Q-Capture Pro 7 (QImaging) software. Quantification of ORO staining was performed off-line using Adobe Photoshop CC5.

Total Plasma Cholesterol: The Wako total cholesterol kit was used to measure plasma cholesterol as previously described[26].

Blood Pressure Measurements:

Tail Cuff method: Systolic blood pressure (SBP) was measured in all mice (prior to WTD administration) and after 16 weeks of feeding using the non-invasive tail cuff plethysmography technique. A MC4000 blood pressure analysis system (Hatteras Instruments) was used with a 37°C pre-heated stage. Mice were placed in restrainers with tails placed through a cuff and onto a heart rate monitor. Mice remained in restraints for 5 mins to allow acclimatisation after which 5 preliminary BP measurement cycles were run to ensure BP was detectable. Ten measurement cycles were then performed to ensure correct readings, the average of the readings for systolic, diastolic and mean arterial blood pressure were recorded.

Blood pressure telemetry: Under isoflurane open circuit anaesthesia (1.5-2.5%) *Apoe*$^{-/-}$ and BPH/*Apoe*$^{-/-}$ mice were implanted with radiotelemetry devices weighing 1.4g and approximately 10mm in length (TA11PA-C10; DataSciences International (DSI), St Paul, USA) as previously described.[27] Following a 10-day recovery from surgery, 1 min averages of pulsatile arterial blood pressure readings and locomotor activity were recorded continuously over a 72-hour period sampled at 100 Hz as previously described.[28] Mean arterial pressure and heart rate were analysed in Labview.[29]

Vessel reactivity:

Preparation of vessels: Mice were ethuanased via CO_2 asphyxiation which was confirmed by cessation of corneal and pedal reflexes. The thoracic aorta was removed and placed in ice-

cold Krebs solution (composition in mM: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5, EDTA 0.026 and glucose 5.5). Perivascular fat and adhering connective tissue were removed from the aorta and it was cut into rings 2 mm in length. Each ring was mounted onto two parallel stainless-steel wires (200 µm in diameter) in a 5 ml chamber of a 4 channel myograph (Mulvaney Myograph, model 610M, JP trading), containing oxygenated Krebs solution and maintained at 37°C. Changes in tension were recorded using a PowerLab 8/35 (ADI Instruments Inc) connected to a computer.

Myograph: Tissues were equilibrated at resting tension for 30mins before undergoing a "normalisation" procedure to determine optimal resting tension and vessel diameter. During the normalisation procedure, tissues are subjected to a series of stretches beginning at wire touch and progressing to tensions of 30mN. Once tissues were set at their optimal resting tension, they were equilibrated for a further 30 min before their viability was tested with a high potassium physiological salt solution, (KPSS, composition in mM: KCl 123, $MgSO_4$ 1.17, KH_2PO_4 2.37, $CaCl_2$ 2.5, EDTA 0.02 and glucose 5.5). Once the contractile response had plateaued, the chamber was rinsed three times with normal Krebs solution and the tissue was allowed to return to baseline. For relaxation curves, tissues were pre-constricted to 50% of the KPSS contraction using PE. Once the contraction had plateaued, cumulative concentration-response curves were acquired to ACh (1nM-10µM) or SNP (0.1nM-10µM) in the presence or absence of L-NAME. Only one protocol was completed in each aortic ring to avoid tolerance.

White Blood Cell Counts: White blood cell (WBC) counts were obtained from blood collected via cardiac puncture immediately following euthanasia. The counts were quantified on a Sysmex XS-1000*i* automated hematology analyser (Japan).

Flow Cytometry: All samples were run and analyzed on a BD FACS Canto II flow cytometer or Fortessa X-20 as stated below. Flow cytometry antibodies were all used at 1:400 dilutions and details of antibodies are available in Supplementary table 1.

T-Cell Populations: Aortas were harvested from the mice following cardiac puncture and flushing with saline. Aortas were digested in Liberase (Sigma-Aldrich, USA, Cat # 5401160001) for 45 mins at 37°C after which the cells were spun down, washed and resuspended in FACS Buffer (1x HBSS + 0.5mM EDTA and 0.05% BSA). Viable cells were gated using a live/dead stain (Ghost violet 510 viability dye, Tonbo Biosciences) on the Canto II where $CD4^-$ T cells were gated as $CD45^+CD3^+$ $CD4^-$ and $CD4^+$ T cells as $CD45^+CD3^+$ $CD4^+$.

Monocyte Populations: Bone marrow and spleen cells were obtained by mashing whole spleen through a 40µm strainer and then lysed using 1x RBC lysis buffer. Blood was collected via cardiac puncture into EDTA-lined tubes and placed directly on ice to prevent leukocyte activation and loss of cell surface CD115. The samples remained on ice or at 4°C for the remainder of the procedure. Red blood cells (RBCs) were lysed, and the washed cells were then stained with CD45, CD115 and Gr1 (Ly6-C/G) for 30 min on ice. The cells were washed, resuspended in FACS buffer and run on a Canto II flow cytometer (Figure 3) or a Fortessa X-20 (Figure 5). Leukocyte populations were identified as previously described.[20, 26, 30] Briefly, viable, single cells were selected on the basis of forward and side scatter characteristics, from which $CD45^+$ leukocytes were selected. Monocytes were identified as being $CD115^+$ with Ly6-

C^{hi} monocytes identified as Gr1^{+} and Ly6-C^{lo} monocytes identified as Gr1^{-}. Neutrophils were identified as CD115^{-}Gr1^{+}.

Hematopoietic stem and progenitor cells Populations: Organs were collected and processed as mentioned above. Samples were then stained with a cocktail of antibodies (30 mins on ice) before analysis by flow cytometry as previously described[20, 26, 30]. Briefly, lineage committed cells were identified as (CD45R, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4 and Ly6-C/G, all FITC positive) with antibodies against Sca1 and cKit to identify myeloid progenitor cells (Lineage^{-}, Sca1^{-}, cKit^{+}) and HSPCs (Lineage^{-}, Sca1^{+}, cKit^{+}). GMPs were identified as (Lineage^{-}, Sca1^{-}, cKit^{+}, Fcγ^{hi}).

Proliferating stem and progenitor cells were assessed by intracellular DAPI (1:1000) staining performed following fixation and permeablization using a BD Fix/Perm kit (BD Biosciences, USA, Cat #. 554722). Samples were run on the Canto II (Figure 3) or Fortessa X-20 (Figure 5).

Bone marrow niche cells: A cocktail of antibodies were used to stain for osteoblast and endothelial cells containing lineage committed cells (CD45R, CD11b, CD3e, TER-119, CD8, CD4, CD45 and Ly-6G; all FITC; eBioscience), as well as Sca1-PB, CD51-PE, F4/80-PE/Cy7 and CD31-PerCP (Biolegend). Osteoblasts were identified as Lin^{-}Sca1^{-}F4/80^{-}CD31^{-}CD51^{+}. Endothelial cells were identified as Lin^{-}Sca1^{-}F4/80^{-}CD31^{+}CD51^{-}. Samples were run on the Fortessa X-20.

Bone Analysis

Processing: Femurs were harvested, fixed in 2% PFA overnight, and immersed in 0.5M EDTA in PBS with 1% PFA for 14 days for decalcification prior to paraffin embedding. The paraffin-embedded sections (5µm thickness) were deparaffinized, rehydrated, and stained.

Bone H&E staining: Sections were in Mayer's hematoxylin for 8 mins followed by a 2 min incubation in scott's tap water. Slides were then stained in eosin for 6 mins, dehydrated, cleared in xylene and mounted with DPX.

Bone Tyrosine Hydroxylase staining: Sections were deparaffinised in histolene and dehydrated using an ethanol gradient. Antigen retrieval was performed by immersing sections in 10mM EDTA at 90°C for 9 minutes in a pressure vessel. Slides were allowed to cool to room temperature. Sections were incubated in 3% H_2O_2 in methanol to quench endogenous peroxidases. Next, the sections were incubated in 10% normal goat serum for one hour followed by Tyrosine hydroxylase (rabbit anti-mouse) primary antibody (1:100, Millipore; AB9983) incubation overnight at 4°C. Following this, sections were incubated with biotinylated goat anti-Rabbit Secondary antibody (1:200; BA-1000 Vector Labs) for 1 hour at room temperature. Sections were then treated with an Avidin-biotin complex (Vectastain ABC HRP kit; PK-4000; Vector Labs) for 30mins followed by DAB treatment (SK-4100; Vector Labs) for 5mins. After washing samples were mounted with DPX mounting media.

Neutrophil derived MMP9 effect on HSPC CXCR4:

Isolation of bone marrow neutrophils: Neutrophils were isolated from the BM of C57BL/6 wildtype mice using the Ficoll method. Cells were overlaid on 3 mL of Ficoll-Paque Plus solution (GE Healthcare), and centrifuged for 20 min with no brakes at 600 g, at room temperature. Cell pellets were washed with PBS and RBC lysed for 1 min. Cells were then re-suspended in media for use. Further details can be found in the supplementary information.

Noradrenaline activation of neutrophils: The catecholamine was prepared by the dissolution of crystalline noradrenaline (NA; Sigma) in PBS. Neutrophils were stimulated with 10 μM of NA for 24 h. To assess the contribution of the β_2-adrenoreceptor to neutrophil responses to NA, we pre-treated (30 mins) the neutrophil suspension with ICI-118551 (100μM) and then co-incubated with NA and ICI-118551 for 24 h (10μM and 100μM respectively).

Treatment of HSPCs with activated neutrophil supernatant: BM suspension from flushed bones (described above) were incubated in the supernatant of neutrophils treated with 0 or 10 μM of NA for 45 mins at 37°C. To test the activity of MMP9, supernatant from NA treated neutrophils was pre-incubated with an MMP inhibitor (50μM; ab142180; Abcam, AUS) for 45 mins prior to incubation with BM suspension. BM was incubated for a further 45 mins in the presence of the MMP inhibitor. Following treatment with supernatant the suspension was washed with FACS buffer and cells were stained to identify HSPCs and CXCR4 expression. The following cocktail was used at 1:400 dilution for each antibody; lineage markers (CD45R, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4 and Ly6-C/G, all FITC positive) with antibodies against Sca1 and cKit to identify HSPCs (Lineage^{-}, Sca1^{+}, cKit^{+}). A CXCR4 antibody was used to determine the surface expression on HSPCs (as Geometric-Mean). Samples were run on the Fortessa X-20 and data were analysed using FlowJo (TreeStar).

High performance liquid chromatography (HPLC) for Plasma Catecholamines: Noradrenaline was extracted from plasma using alumina adsorption and was quantified using high performance liquid chromatography with coulometric detection as previously described.[31]

Zymography: To assess the effects of pressure on the gelatinase MMP9, gelatin zymography was performed on BM extracellular fluid, isolated by collecting the supernatant from flushed BM. Briefly, BM extracellular fluid samples were aliquoted on to 7.5% acrylamide, 0.5% gelatin gels and electrophoresed. Following electrophoresis, gels were washed and incubated overnight in incubation buffer at 37°C. 0.1% Coomassie Blue solution containing 40% 2-propanol was used to stain the gels. Further details can be found in the supplementary information.

RNA isolation, cDNA synthesis and qRT-PCR: Total RNA from BM cells was extracted using QIAGEN RNeasy mini kit and cDNA was synthesized using a Tetro cDNA kit (Bioline). qRT-PCR was assessed in real time with a 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Core Reagents (Agilent Technologies) and normalized to *GAPDH*. All primer sequences can be found in Supplementary Table 2.

Statistics: Data are presented as mean ± SEM (unless stated otherwise) and were analyzed using the two-tailed Student t-test or One-way ANOVA where appropriate. Analysis of baseline and final blood pressure between strains was analysed using a two-way ANOVA with the factors strain (P_{strain}) and time (P_{time}) followed by a Sidak post-hoc test to account for multiple

comparisons. A $P<0.05$ was considered significant. All tests were performed using the Prism software (GraphPad Software, Inc., La Jolla, CA).