SUPPLEMENTAL MATERIAL

Matrix Metalloproteinase-2 Knockout Prevents Angiotensin II-Induced vascular injury

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Materials and Methods

Experimental design

The study was approved by the Animal Care Committee of the Lady Davis Institute for medical research and McGill University, and followed recommendations of the Canadian Council of Animal Care. Ten to 12-week-old male C57BL/6J wild-type (WT) mice (Harlan laboratories, Indianapolis, IN, USA) and *Mmp2* knockout (*Mmp2*$^{-/-}$) mice (generously provided by Dr. Shigeyoshi Itohara[1] and produced at the Lady Davis Institute for medical research) were anesthetized with 3% isoflurane mixed with O_2 at 1 L/min. The depth of anesthesia was confirmed by the rear foot squeezing. The non-steroidal anti-inflammatory drug carprofen (20 mg/Kg) was administered SC to minimize the post-operation pain, and then surgically implanted SC with ALZET osmotic mini pumps (Model 1002, Durect Corporation, Cupertino, CA) infusing angiotensin II (1000 ng/kg/min) for 14 days, as recommended by the manufacturer. Control mice underwent sham surgery. Blood pressure (BP) was determined by telemetry as previously described.[2] In brief, 10-week-old mice were anesthetized with isoflurane and injected with carprofen as above, surgically instrumented with PA-C10 telemetry transmitters as recommended by the manufacturer (Data Sciences International, St. Paul, MN). Mice were allowed to recover for 7 to 10 days and carprofen administered as above once a day for the first two days, and then treated as described above. BP was determined every 5 min for 10 sec from two days before angiotensin II mini pump or sham surgery until the mice were sacrificed.

In order to examine the role of MMP2 in angiotensin II signaling in vascular smooth muscle cells (VSMCs), another set of 8-9 week-old male WT and *Mmp2*$^{-/-}$ mice were used to isolate the VSMCs. WT and *Mmp2*$^{-/-}$ VSMCs were starved in DMEM high glucose supplemented with 0.2% fetal bovine serum (FBS, qualified, Canada origin, Life Technologies, Burlington, ON, Canada) for 24 h and, treated with vehicle or 100 nmol/L angiotensin II for 5 min, washed with ice-cold phosphate-buffered saline (PBS), lysed with RIPA buffer (125 mmol/L NaCl, 0.5% sodium desoxycholate, 1 mmol/L sodium pyrophosphate, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.1% SDS and 50 mmol/L Tris, pH 7.4) supplemented with 2 mmol/L sodium orthovanadate, 20 mmol/L sodium fluoride, 0.1% protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and the levels of phosphorylation of epidermal growth factor receptor (EGFR) and of p44/42 mitogen-activated protein kinase (extracellular-signal-regulated kinase 1/2, ERK1/2) were determined by Western blot.

In another subset of mice, CD45^{+} immune cells were isolated from thoracic aorta with the surrounding perivascular adipose tissue (PVAT) and *Mmp2* expression was determined by reverse transcription (RT)-quantitative PCR (qPCR).

In order to elucidate the contribution of immune and vascular cell MMP2 to angiotensin II-induced vascular injury, irradiation-bone marrow transplantation experiments were performed using 8-10 week-old male WT and *Mmp2*$^{-/-}$ mice as bone marrow donor and recipient. Bone marrow (BM) cells isolated from WT or *Mmp2*$^{-/-}$ mice was transplanted into γ-irradiated WT (WT→WT or *Mmp2*$^{-/-}$→WT) or *Mmp2*$^{-/-}$ (WT→*Mmp2*$^{-/-}$ or *Mmp2*$^{-/-}$→*Mmp2*$^{-/-}$) mice as described previously.[3] Four weeks after transplantation, mice were instrumented or not with PA-C10 telemetry transmitters for BP determination, and 7-10 days later BP was determined for 2 consecutive days before and during the 14-day period of treatment with or without angiotensin II and studied as above. BP was also determined in an additional group of BM-transplanted mice infused with L-norepinephrine (4.17 μg/Kg/min) using ALZET osmotic pumps for 14 days as previously described.[4]

Collection of tissues

At the end of the protocol, mice were weighed and then anesthetized with isoflurane as above. The mesenteric artery (MA) vascular bed was dissected, and aorta, heart, lung, spleen, liver, kidneys and tibia were harvested in ice-cold phosphate buffered saline (PBS). Tissues were weighed and tibia length determined. Spleen was used for T cell and monocyte profiling. Second-order MA were used for assessment of endothelial function and vessel mechanics. Sections of aorta were embedded in VWR Clear Frozen Section Compound (VWR international, Edmonton, AL, Canada) for determination of ROS generation, expression of vascular cell adhesion molecule 1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1) or monocyte/macrophage and $CD3^+$ T cell infiltration or in paraffin for quantification of collagen content with Sirius red staining. The remaining tissues were frozen in liquid nitrogen and stored at -80°C until used.

In another set of mice, whole thoracic aortas were collected with surrounding perivascular adipose tissue (PVAT), blood was flushed out and tissues were used for determination of *Mmp2* mRNA expression in $CD45^+$ immune cells.

In BM-transplanted mice, blood was also collected by cardiac puncture on EDTA. One hundred µL of blood was used for confirmation of the success of bone marrow reconstitution. Otherwise, blood samples were centrifuged at 1,000 x *g* for 15 min at 4°C to remove blood cells, followed by centrifugation at 10,000 x *g* for 10 min at 4°C to remove platelets. Plasma samples were stored at -80°C until used for MMP2 and pro-MMP9 determinations.

Assessment of Endothelial Function and Vessel Mechanics

Second-order MA, of average lumen size ~220 µm, were dissected and mounted on a pressurized myograph as previously described.[5] Vessels were equilibrated for 45 min at 45 mm Hg intraluminal pressure in Krebs solution (pH 7.4) containing (in mmol/L): 120 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.18 KH_2PO_4, 1.18 $MgSO_4$, 2.5 $CaCl_2$, 0.026 EDTA and 5.5 glucose, and bubbled continuously with 95% air and 5% CO_2. Media and lumen diameters were measured by a computer-based video imaging system (Living Systems Instrumentation, Burlington, Virginia, USA). Contractile responses to cumulative concentrations of norepinephrine (10^{-8} to 10^{-4} mol/L) were determined. Endothelium-dependent and -independent relaxation was assessed by measuring the dilatory responses to cumulative concentrations of acetylcholine (10^{-9} to 10^{-4} mol/L) or sodium nitroprusside (SNP, 10^{-8} to 10^{-4} mol/L), respectively, in vessels precontracted with norepinephrine (5×10^{-5} mol/L). To evaluate the contribution of nitric oxide (NO) to the vascular response, the dose-response curve to acetylcholine was determined before and after a 30-min preincubation with the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-4} mol/L). Thereafter, vessels were perfused with Ca^{2+}-free Krebs solution containing 10 mmol/L EGTA for 30 min to eliminate the vascular tone. Media and lumen diameters were measured at 3, 10, 20, 30, 40, 45, 60, 80, 100, 120 and 140 mm Hg intraluminal pressures. Media cross-sectional area, media/lumen, and stress and strain were calculated as previously described.[6]

Generation of reactive oxygen species (ROS)

Media, adventitia and perivascular adipose tissue (PVAT) ROS production were assessed on 5 µm cryosections of aorta by measuring fluorescence after incubation with the ROS-sensitive fluorescent dye dihydroethidium (DHE, 2 µmol/L) in dark for 1 min at 37°C. Fluorescence was visualized and captured with a fluorescence microscope with a CY3 filter as previously described.[5] DHE fluorescence intensity per total surface area was quantified with ImageJ software (http://rsb.info.nih.gov/ij/) and expressed as fold change relative to control.

Immunofluorescence

Immunofluorescence microscopy was performed on 5-µm-thick cryostat aortic sections. Aortic sections were air-dried for 30 min, fixed for 5 min at room temperature in a mixture of acetone:methanol (1:1) pre-chilled at -20˚C and washed with Tris-buffered saline (TBS) containing 0.1 % Tween-20 (TBST) twice for 10 min. Sections were blocked with TBST containing 10% normal goat serum for 1 h at room temperature and then incubated with a rabbit anti-mouse fibronectin antibody (1:400, EMD Millipore, Billerica, MA, USA), a rat anti-macrophage-specific antigen MOMA-2 (1:50, Abcam, Cambridge, MA,USA), a rabbit anti-VCAM-1 (1:100, Abcam, Cambridge, MA, USA), a goat anti-MCP-1 (1:50, Santa Cruz Biotechnology, Santa Cruz, California USA), or a rat anti-mouse CD3 antibody (1:100, eBiosciences, San Diego, CA) in blocking solution overnight at 4°C. The sections were washed 3 times with TBST and incubated with an Alexa® Fluor 594 goat anti-rabbit antibody (1:400) for fibronectin, an Alexa Fluor® 568 goat anti-rat (1:200) for MOMA-2 and CD3, an Alexa Fluor® 568 goat anti-rabbit (1:200) for VCAM-1 or an Alexa Fluor® 555 donkey anti-goat for MCP-1 (1:200) antibody (all from Life Technologies) in the blocking solution for 1h at room temperature and then washed 3 times with TBST and incubated with 4',6-diamidino-2-phenylindole (DAPI, 6 µM, Life Technologies), and mounted with Fluoromount (Sigma-Aldrich Canada, Oakville, ON, Canada). Images were captured using a fluorescent microscope Leica DM2000 (Leica Microsystems, Richmond Hill, ON, Canada) and quantified with ImageJ software. The expression of VCAM-1 and MCP-1 was determined in aortic wall and was expressed as relative fluorescence unit (RFU) per μm^2. The expression of fibronectin was determined in aortic wall and was expressed as fold change relative to control. Monocyte/macrophage infiltration was analyzed in aortic PVAT by determining the area of MOMA-2 staining, which was then expressed as % or studied area. CD3 infiltration was quantified aortic PVAT as the number of cells detected per μm^2.

Collagen content

Sections (5 µm) of paraffin-embedded tissues were stained with Sirius red to determine aortic collagen type I and III content as previously described.[4, 7] Images were acquired by light microscopy using a Leica DM2000 microscope, and analyzed by color RGB thresholding using Northern Eclipse software (EMPIX Imaging, Missisauga, ON, Canada). Aortic wall collagen fraction was defined as the ratio of the media stained area to the total media area and expressed as a percentage.

Flow cytometry profiling of splenic T cells and monocytes

Profile of T cells and monocyte subtypes was determined by flow cytometry as previously.[7] Single splenocyte suspension was obtained by releasing the splenocytes by forcing pieces of spleen through a 70 µm nylon mesh cell strainer (BD Biosciences, Durham, NC, USA) pre-wet with PBS supplemented with 5% FBS with the back of a 3 mL syringe plunger. The cell strainer was washed with PBS/5% FBS to flush the cells through the nylon mesh. The two previous steps were repeated until only connective tissue remained in the cell strainer. Cells were centrifuged at 300*g* for 10 min at room temperature. Cells were resuspended in 5 mL of Red Blood Cell lysis buffer (Sigma-Aldrich Canada) and incubated at room temperature for 3 min with occasional gentle mixing to eliminate red blood cells. The mixture was diluted with 30 mL of PBS/5% FBS, filtered through a 70 µm nylon mesh cell strainer, and centrifuged at 300 *g* for 5 min at RT. Cells were resuspended in 2 mL of PBS/5% FBS and counted using a Z2 Coulter® Counter (Beckman-Coulter, Mississauga, ON, Canada). Two million cells were stained with a fixable viability dye eFluor® 506 (eBioscience, San Diego, CA, USA) in PBS, incubated with rat anti-mouse CD16/CD32 Fc

receptor block (clone 2.4G2, BD Biosciences), and stained with specific antibodies in PBS/5% FBS, and analyzed by flow cytometry. Antibodies are listed in Table S1 for profiling of T cells and Table S2 for profiling of monocytes. Flow cytometry was performed on the BD LSRFortessa cell analyzer (BD Biosciences). Fluorescence minus one controls were used to determine fluorescence background and positivity. Data analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Isolation of aortic perivascular fat infiltrating immune cells

Thoracic aortas with the surrounding PVAT were sliced in small pieces and digested in 800 µL of enzyme digestion medium at 37°C for 1 h in a 1.5 mL conical tube with gentle agitation in a hybridization oven (VWR International, LLC Radnor, PA, USA). The enzyme digestion medium was constituted of RPMI medium 1640 (Life Technologies, Burlington, Ontario, Canada) supplemented with 1 mg/ml of collagenase A (0.229 U/mg), 500 U/ml of collagenase type 2 (290 U/mg), 2 U/ml of elastase (4.41 U/mgP) and 250 µg/ml of soybean trypsin inhibitor (1 mg SI inhibits 2 mg TRL) that was filtered with 0.22 µm Millex GP filter unit (EMD Millipore, Billerica, MA, USA). Collagenase A was obtained from Roche Diagnostics GmbH (Mannheim, Germany) and all the other enzymes from Worthington Biochemical Corporation (Lakewood, NJ, USA). At the end of the digestion, the cell suspension was passed through a 70 µm BD Falcon nylon mesh cell strainer fitted on top of a 1.5 mL tube. After centrifugation at 410 x *g* for 5 min at 4°C, the pellet of cells was suspended in 50 µl of staining buffer for 15 min at 4°C. Staining buffer consists of RPMI 1640 medium supplemented with 5% FBS with 4 µg/ml of Brilliant Violet 785® anti-mouse CD45 antibody (Biolegend, San Diego, CA, USA). The collected cells were centrifuged at 410 x *g* for 5 min at 4°C and the pellet of cells was resuspended in 300 µl of PBS. $CD45^+$ cells were isolated by fluorescence-activated cell sorting with a BD FACSAria Fusion flow cytometer (BD Biosciences) using the band pass filter 780/60 nm. The $CD45^+$ sorted cells were collected in 1.5 mL tube containing 200 µl of FBS and stored on ice until RNA isolation.

Evaluation of *Mmp2* expression

The expression of *Mmp2* and ribosomal protein S16 (*Rps16*) mRNA was determined in aortic PVAT $CD45^+$ immune cells by reverse transcription RT-qPCR. The suspension of cells was centrifuged at 410 x *g* for 5 min at 4°C, the pellet of cells was resuspended in 500 µl of Lysis/Binding Buffer and processed for total RNA extraction using the mirVana miRNA isolation kit (Life Technologies) following manufacturer instructions. RNA concentration was measured using a Nanodrop spectrophotometer ND-100 V3.1.2 (Thermo Fisher Scientific, Wilmington, DE). One hundred ng of total RNA was reverse-transcribed with the Quantitect RT kit (Qiagen, Foster City, CA, USA). The qPCR was performed using the SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Mississauga, ON, Canada) with the Mx3005P real-time PCR cycler (Agilent Technologies, Santa Clara, CA). Oligonucleotides designed for *Mmp2* and *Rps16* are listed in Table S3. The qPCR conditions used were 2 min at 96 °C, followed by 40 cycles of 5 sec at 96°C and 30 sec at 58°C. Results were normalized with *Rps16* and expressed as fold change over control.

VSMC isolation, culture and treatment

VSMCs were isolated as previously described with some modification.[8] Three mice per VSMC isolation were used. Mice were anesthetized with isoflurane as above and the MA vascular beds were dissected and collected and stored in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) cell culture medium (Life Technologies) at room temperature until further processing for VSMC isolation. MA vascular beds were dissected quickly in DMEM/F12 medium

to remove the fat under a dissecting microscope. MA vascular beds were digested in 12.5 mL of DMEM/F12 medium containing 25 mg bovine serum albumin (Probumin media grade, EMD Millipore), 25 mg of collagenase type 2 (280 units/mg dry weight, Worthington Biochemical Corporation, Lakewood, NJ), 1.5 mg elastase (4.92 units/mg protein, Worthington Biochemical Corporation) and 4.5 mg soybean trypsin inhibitor (Sigma-Aldrich Canada) at 37ºC for 30 min in a 50 mL conical tube with gentle agitation in a hybridization oven (VWR International, LLC Radnor, PA, USA). Digestion efficiency was improved by pipetting up and down the tissue with a 1-mL pipette after the first 10 min of digestion and then after every 5 min. At the end of the digestion, debris were removed by passing the digesta through a 100 μm Falcon cell strainers fitted on top of a 50 mL conical tube. The cell strainers was washed with 5 mL of DMEM/F12 medium. VSMC suspension was centrifuged at 300 *g* for 5 min at room temperature. VSMCs were resuspended in 1 mL of culture medium (DMEM high glucose medium supplemented with 10 % heat inactivated FBS, 100 Units/mL of penicillin - streptomycin and 2.8 mmol/L of L-glutamine (all from Life Technologies). A cell suspension aliquot was mixed 1:1 with a 0.4% trypan blue solution (Life Technologies) and viable cells were counted with a hemocytometer. VSMC were seed in 25 cm^2 Falcon Primaria cell culture flasks at a density of 40,000 cells/cm^2 and kept in a humidified 37°C incubator gassed with 5% CO_2. Culture medium was changed every 48 h. When cells reached 70-80% confluence, they were trypsinized, washed with culture media and passaged 1:3 into 75 cm^2 Falcon Primaria cell culture flasks (passage 1). VSMC were passaged as above one more time (passage 2) and then passaged at a density of 12,411 cells/cm^2 into 60 mm Falcon Primaria petri dishes (passage 3). When cells reached 70-80% confluence, they were starved in DMEM high glucose supplemented with 0.2% FBS for 24 h and, treated with vehicle or 100 nM angiotensin II for 5 min, the culture medium removed rapidly and cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with 150 μL of RIPA buffer (125 mmol/L NaCl, 0.5% sodium desoxycholate, 1 mmol/L sodium pyrophosphate, 0.1 mmol/L EDTA, 0.1 mM EGTA, 0.1% SDS and 50 mmol/L Tris, pH 7.4) supplemented with 2 mmol/L sodium orthovanadate, 20 mM sodium fluoride, 0.1% protease inhibitor cocktail (Roche, Indianapolis, IN, USA) per 60 mm petri dish. Lysates were sonicated to reduce the viscosity using a sonicator VCX-400W equipped with a probe model CV20 (Vibra cell, Danbury, CT, USA) at an amplitude of 20% for 10 sec on ice. Lysates were centrifuged at 14,000 *g* for 15 min at 4°C and the protein concentration in the supernatant was determined using the DC Protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein extracts were stored at -80°C until used.

Western-Blotting

The levels of phosphorylation of epidermal growth factor receptor (EGFR) and of p44/42 mitogen-activated protein kinase (extracellular-signal-regulated kinase 1/2, ERK1/2) were determined by Western blot. Proteins (15-20 μg) were separated by SDS-PAGE (10% 37.5:1 acrylamide/bis-acrylamide, Bio-Rad Laboratories), transferred to nitrocellulose Hyband ECL membrane (GE Healthcare Life Sciences, Mississauga, ON, Canada) for EGFR or Immuno-Blot polyvinylidene fluoride (PDVF) membrane (Bio-Rad Laboratories) for ERK1/2. Membranes were blocked with 5% pasteurized skim milk powder for 1 h at room temperature and incubated with rabbit anti-pY1086 EGFR (1:1000, Life Technologies) or goat anti-phospho-ERK1/2 (Thr202/ Tyr204, 1:1000, Cell Signaling Technology, Danvers, MA, USA) antibody overnight at 4°C. Membranes were washed 5 times 5 min with TBST, blocked with 5% milk and incubated with horseradish peroxidase (HRP) goat anti-rabbit (1/1000) or HRP rabbit anti-goat (1/1000) antibody (all from Bio-Rad Laboratories) as appropriate for 1 h at room temperature. Membranes were washed 5 times 5 min with TBST and signals were revealed by chemiluminescence (Clarity™ Western ECL

Blotting Substrate, Bio-Rad Laboratories) with the molecular imager ChemiDoc™ MP System (Bio-Rad Laboratories). Membranes were stripped with Re-Blot Plus Strong Solution (10X) (Temecula, California USA) for 6 min at room temperature, blocked with 5% milk and incubated with goat-anti-human EGFR (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA) or rabbit anti-p44/42 MAPK (Erk1/2, 1:1000, Cell Signaling Technology) antibody as appropriate overnight at 4°C. Membranes were washed 3 times 5 min with TBST, blocked with 5% milk and incubated with HRP goat anti-rabbit (1/1000, Bio-Rad Laboratories) or HRP rabbit anti-goat (1/1000, Bio-Rad Laboratories) antibody as appropriate for 1 h at room temperature. Membrane were washed 3 times with TBST and signals were revealed as above. Signals were quantified using Image Lab 5.2 software (Bio-Rad Laboratories). The levels of phosphorylation were reported as the ratio of the relative amount of phospho-protein on the relative amount of total protein.

Bone marrow cell transplantation

Two weeks before bone marrow cell transplantation, 8-10 week-old male recipient WT and *Mmp2*$^{-/-}$ mice were provided autoclaved tap water acidified to pH 2.5-3.0 with concentrated HCl and supplemented with 0.15 mg/mL enrofloxacin (Baytril, Bayer Inc., Toronto, ON, Canada). On the day of bone marrow cell transplantation, recipient mice were anesthetized by injection of 10 µL/10 g of rodent cocktail constituted of 50 mg/kg ketamine (Vetalar, Bioniche Animal Health Canada INC., Belleville, ON, Canada), 5 mg/kg xylazine (Rompun, Bayer Health Care, Toronto, ON, Canada) and 1 mg/kg acepromazine maleate (Atravet, Boehringer Ingelheim (Canada) Ltd., Burlington, ON, Canada), a subcutaneous injection of 1 ml of sterile saline (0.9% NaCl) was administrated and mice were lethally irradiated with a single dose of 10 Gy (0.2 Gy/min) total body irradiation using a Cobalt-60 (^{60}Co) teletherapy machine Theratron-780 (MDS Nordion, Ottawa, Canada). The animals were be putted in their cage in a warm incubator until full recovery from the anesthesia (~3 h). During this period, bone marrow cells were isolated from one 8-10 week-old male donor WT or *Mmp2*$^{-/-}$ mouse per 4 recipient mice. Donor mice were euthanized by CO_2 inhalation following by cervical dislocation and the femurs and tibia were quickly dissected and placed in RPMI 1640 (with GlutaMAX™ Supplement, Life Technologies) culture medium containing 2% FBS, 10 units/ml heparin and 100 units/mL penicillin-streptomycin. (Life technologies). Bone marrow cells were obtained by flushing femurs and tibia using a 25G 5/8 needle with culture medium. The cell suspension was filtered through a sterile Falcon 70 µm nylon mesh cell strainer and collect into a 50 ml conical tube. The volume was adjusted to 50 ml with the culture medium and centrifuge at 900 *g* for 10 min. Bone marrow cells were washed twice with 50 ml of serum-free culture medium. Bone marrow cells were suspended in 1 mL of PBS, the number alive cells determined with the vital stain trypan blue and the cell number was adjusted to 10^7 cells/150 µl of PBS. Recipient mice were immediately reconstituted with 150 µl of WT or *Mmp2*$^{-/-}$ bone marrow cell suspension by a retro-orbital injection. Recipient mice were returned to their cages and provided sterile acidified tap water supplemented with antibiotics for 4 weeks of recovery before used as described in Experimental design section and sterile acidified tap water until the end of the study. The success of bone marrow reconstitution was confirmed at the end of the study by qPCR.

Confirmation of bone marrow reconstitution

The amount of *Mmp2* and *neo* genes (neomycin resistance gene encoding aminoglycoside phosphotransferase that is contained in *Mmp2* knockout gene) in circulating mononuclear cell genomic DNA was determined by qPCR as follows. Genomic DNA was extracted from 100 µL of blood using PureLink™ Genomic DNA Kit (Life Technologies, USA) according to the

manufacturer's protocol. DNA concentration was measured with a NanoDrop spectrophotometer ND-100 V3.1.2 (Thermo Fisher Scientific) and stored at −20°C until used. qPCR was performed with 0.36 ng of genomic DNA using SsoFast EvaGreen Supermix (Bio-Rad Laboratories) with the Mx3005P real-time PCR cycler (Agilent Technologies). The qPCR conditions used were 2 min at 98°C, followed by 40 cycles of 5 sec at 98°C and 30 sec at 58°C. *Mmp2* and *neo* qPCR results were normalized to the amount of beta-actin (*Actb*) gene and expressed as % of WT and *Mmp2*$^{-/-}$ reference control, respectively. Primers are described in Table S4.

Plasma levels of MMP2 and pro-MMP9

Plasma levels of MMP2 and pro-MMP9 were measured using MILLIPLEX® MAP mouse MMP Magnetic Bead Panel 1 and 2 (EMD Millipore), respectively, on a Bio-Plex 200 (Bio-Rad Laboratories).

Data Analysis

Results are presented as means ± SEM. Data were compared with two-way analysis of variance (ANOVA) or two-way ANOVA for repeated measures, with all ANOVA tests followed by a Student–Newman–Keuls *post-hoc* test, or with an unpaired *t*-test, as appropriate. $P<0.05$ was considered statistically significant.

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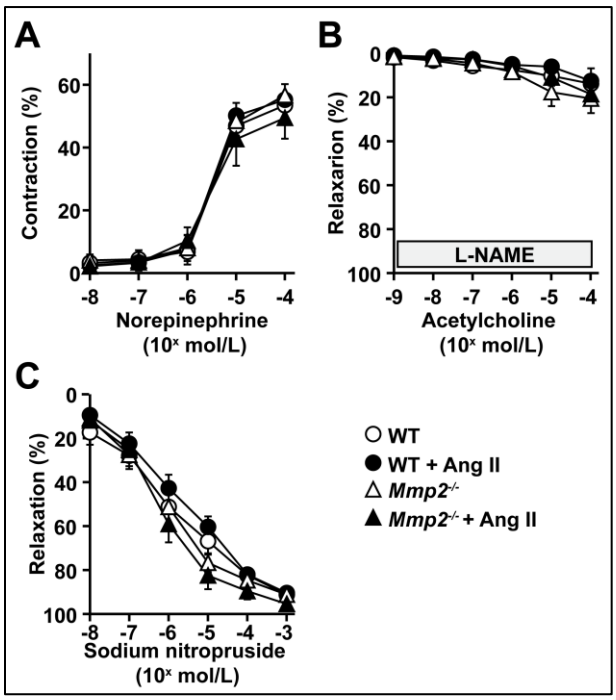

Figure S1. Mesenteric small artery norepinephrine contractile responses (**A**), acetylcholine relaxation responses in absence of nitric oxide synthase (**B**) and sodium nitroprusside relaxation responses (**C**) were unaffected by angiotensin (Ang) II infusion or *Mmp2* knockout. These parameters were determined in wild-type (WT) and *Mmp2* knockout (*Mmp2*$^{-/-}$) mice infused or not with Ang II for 14 days. Values are means ± SEM. Number of samples per group for **A**: WT and *Mmp2*$^{-/-}$ = 6 and Ang II treated groups = 5. For **B and C**: WT = 7, WT + Ang II = 6 and *Mmp2*$^{-/-}$ and *Mmp2*$^{-/-}$ + Ang II = 8.

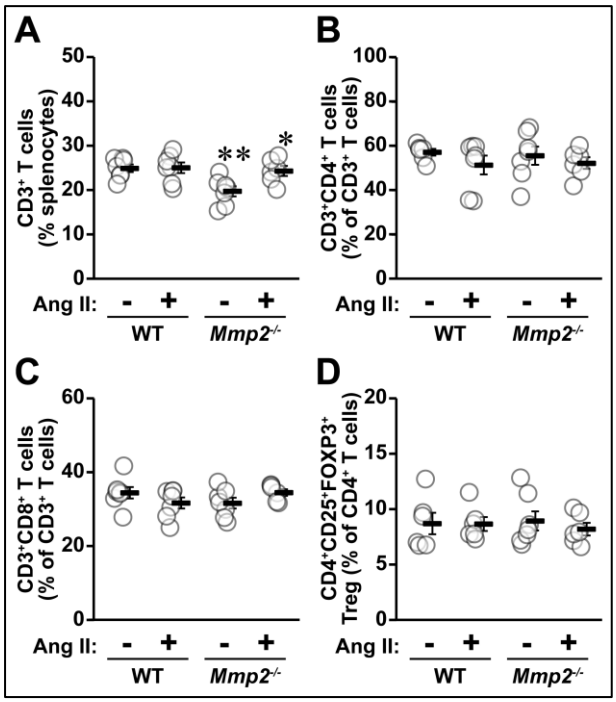

Figure S2. *Mmp2* gene deletion reduced the fraction of pan ($CD3^+$) T cells (**A**), but not T helper ($CD3^+CD4^+$) cells (**B**), cytotoxic ($CD3^+CD8^+$) T cells (**C**) and T regulatory cells ($CD3^+CD4^+CD25^+FOXP3^+$) (**D**) in the spleen. These parameters were determined in wild-type (WT) and *Mmp2* knockout (*Mmp2*$^{-/-}$) mice infused or not with Ang II for 14 days. Values are means ± SEM. Number of samples per group for **A** and **C**: *Mmp2*$^{-/-}$ + Ang II = 6 and other groups = 7. For **B** and **D**: WT and *Mmp2*$^{-/-}$ + Ang II = 6 and other groups = 7. Data were analyzed using a two-way ANOVA followed by a Student-Newman-Keuls post hoc test. **P*<0.05 and ***P*<0.01 vs. their respective controls.

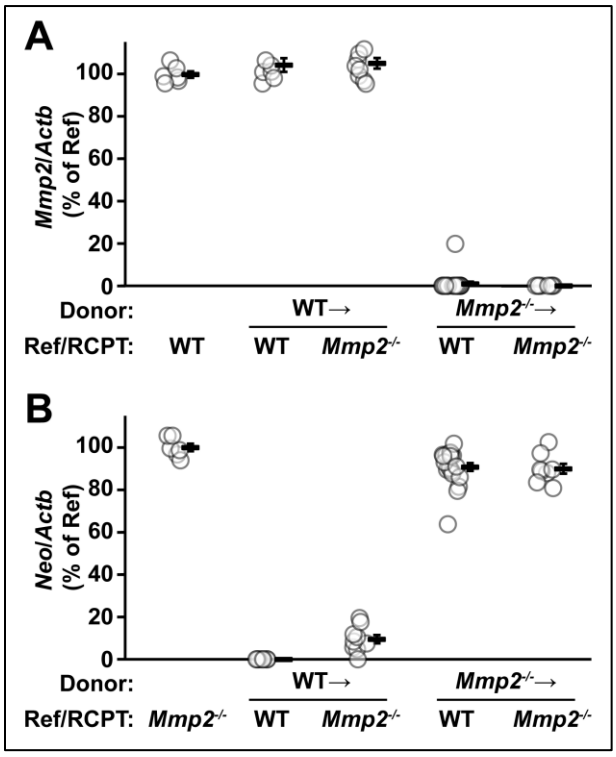

Figure S3. γ-irradiated wild-type (WT) and *Mmp2*$^{-/-}$ recipient (RCPT) mice were efficiently reconstituted by bone marrow transplantation from WT and *Mmp2*$^{-/-}$ donor mice. The amount of *Mmp2* gene (**A**) and *neo* (**B**, neomycin resistance gene encoding aminoglycoside phosphotransferase that is contained in *Mmp2* knockout gene) gene was determined by quantitative PCR in circulating mononuclear cell genomic DNA of γ-irradiated WT and *Mmp2*$^{-/-}$ RCPT mice transplanted with bone marrow of WT (WT→WT and WT→*Mmp2*$^{-/-}$) and *Mmp2*$^{-/-}$ (*Mmp2*$^{-/-}$→WT and *Mmp2*$^{-/-}$→*Mmp2*$^{-/-}$) donor mice. *Mmp2* and *neo* gene r results were normalized to the amount of beta-actin (*Actb*) gene and expressed as % of WT and *Mmp2*$^{-/-}$ reference (Ref) control, respectively. Values are means ± SEM, Number of samples per group: WT and *Mmp2*$^{-/-}$ Ref = 6, WT→WT = 7, WT→*Mmp2*$^{-/-}$ = 9, *Mmp2*$^{-/-}$→WT = 20 and *Mmp2*$^{-/-}$→*Mmp2*$^{-/-}$ = 8.

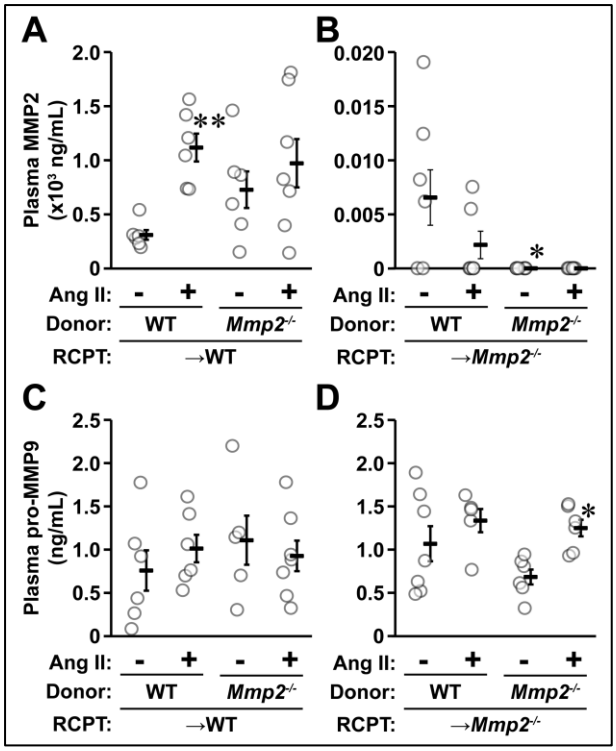

Figure S4. Plasma MMP2 and pro-MMP9 in bone marrow transplanted γ-irradiated recipient wild-type (WT) and *Mmp2*$^{-/-}$ mice. Bone marrow from WT and *Mmp2*$^{-/-}$ donor mice was transplanted into γ-irradiated WT (**A** and **C**, WT→WT and *Mmp2*$^{-/-}$→WT) and *Mmp2*$^{-/-}$ (**B** and **D**, WT→ *Mmp2*$^{-/-}$and *Mmp2*$^{-/-}$→*Mmp2*$^{-/-}$) recipient (RCPT) mice. One month later, mice were treated or not with Ang II for 14 days and MMP2 (**A** and **B**) and pro-MMP9 (**C** and **D**) were determined in the plasma. Values are means ± SEM. Number of samples per group for **A**: *Mmp2*$^{-/-}$→WT + Ang II = 6 and other groups = 7. For **B**: WT→WT + Ang II = 6, *Mmp2*$^{-/-}$→WT + Ang II = 7 and other groups = 7. For **C**: *Mmp2*$^{-/-}$→WT = 5, *Mmp2*$^{-/-}$→WT + Ang II = 7 and other groups = 6. For **D**: WT→WT = 7, WT→WT + Ang II = 5, and other groups = 6. Data were analyzed using a two-way ANOVA followed by a Student-Newman-Keuls *post hoc* test. **P*<0.05 and ***P*<0.01 vs. respective control.

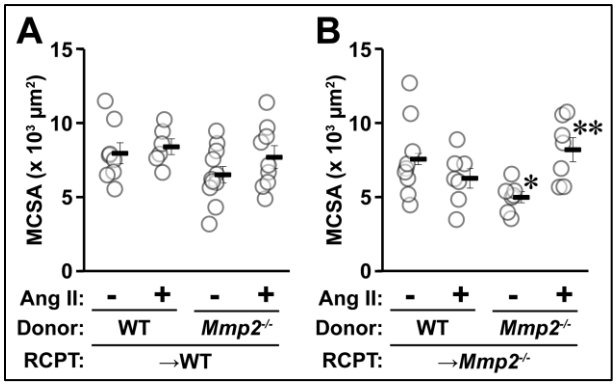

Figure S5. Mesenteric artery media cross-sectional area (MCSA) was smaller and increased by Ang II in γ-irradiated *Mmp2*$^{-/-}$ recipient (RCPT) mice that have received bone marrow from *Mmp2*$^{-/-}$ donor mice. Role of vascular tissue and bone marrow-derived cell MMP2 in angiotensin (Ang) II-induced vascular remodeling. Bone marrow from wild-type (WT) and *Mmp2*$^{-/-}$ donor mice was transplanted into γ-irradiated WT (**A**, WT→WT and *Mmp2*$^{-/-}$→WT) and *Mmp2*$^{-/-}$ (**B**, WT→*Mmp2*$^{-/-}$ and *Mmp2*$^{-/-}$ →*Mmp2*$^{-/-}$) recipient (RCPT) mice. One month later, mice were treated or not with Ang II for 14 days. Remodeling of MCSA of mesenteric arteries was determined by pressurized myography with an intraluminal pressure of 45 mm Hg. Values are means ± SEM. Number of samples per group for **A**: WT→WT = 8, WT→WT + Ang II = 6, *Mmp2*$^{-/-}$→WT = 11 and *Mmp2*$^{-/-}$→WT + Ang II = 9. For **B**: WT→ *Mmp2*$^{-/-}$ = 9 and other groups = 7. Data were analyzed using a two-way ANOVA followed by a Student-Newman-Keuls *post hoc* test. **P*<0.05 and ***P*<0.01 vs. respective control.

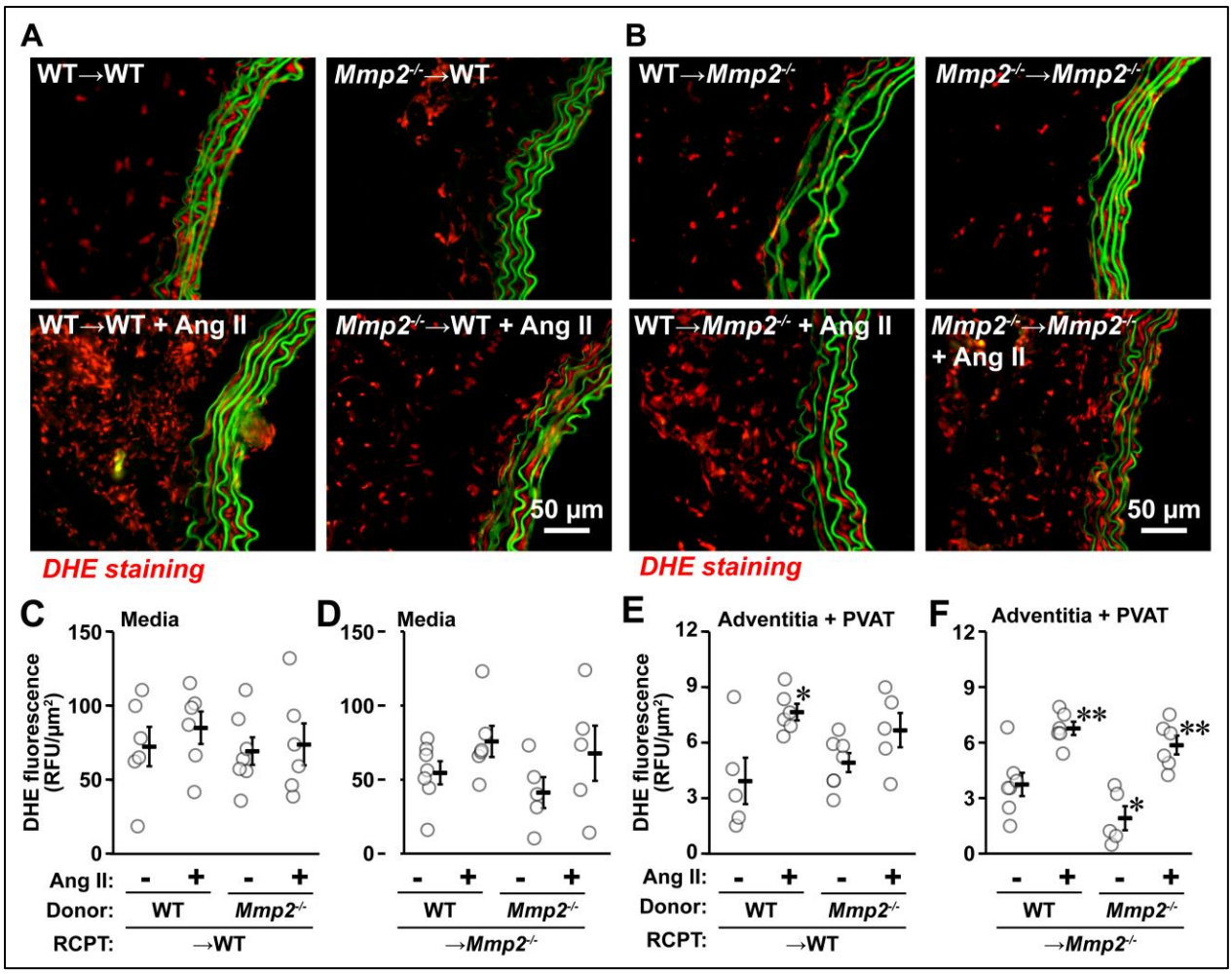

Figure S6. Absence of immune cell MMP2 blunted angiotensin (Ang) II-induced reactive oxygen species (ROS) generation in aortic adventitia and perivascular adipose tissue (PVAT) . Bone marrow from wild-type (WT) and *Mmp2*$^{-/-}$ donor mice was transplanted into γ-irradiated WT (**A, C** and **E**, WT→WT and *Mmp2*$^{-/-}$→WT) and *Mmp2*$^{-/-}$ (**B, D** and **F**, WT→ *Mmp2*$^{-/-}$and *Mmp2*$^{-/-}$ →*Mmp2*$^{-/-}$) recipient (RCPT) mice. One month later, mice were treated or not with Ang II for 14 days and ROS generation was determined in the aorta by dihydroethidium (DHE) staining in the media (**C** and **D**), and adventitia and PVAT (**E** and **F**). Values are means ± SEM. Number of samples per group for **A**: *Mmp2*$^{-/-}$→WT = 7 and other groups = 6. For **B**: *Mmp2*$^{-/-}$→WT = 7, *Mmp2*$^{-/-}$→WT + Ang II = 6 and other groups = 5. For **C**: WT→WT + Ang II = 6, *Mmp2*$^{-/-}$→WT = 7 and other groups = 5. For **D**: WT→*Mmp2*$^{-/-}$ = 7, *Mmp2*$^{-/-}$→*Mmp2*$^{-/-}$ = 5 and other groups = 6. Data were analyzed using a two-way ANOVA followed by a Student-Newman-Keuls *post hoc* test. **P*<0.05 and ***P*<0.001 vs. respective control.

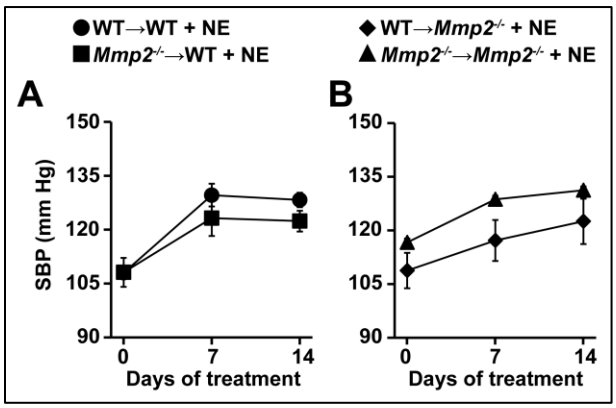

Figure S7. Norepinephrine (NE)-induced BP elevation was unaffected by lack of MMP2 in immune or vascular cells. Bone marrow from wild-type (WT) and *Mmp2*$^{-/-}$ donor mice was transplanted into γ-irradiated WT (**A**, WT→WT and *Mmp2*$^{-/-}$→WT) and *Mmp2*$^{-/-}$ (**B**, WT→ *Mmp2*$^{-/-}$and *Mmp2*$^{-/-}$→*Mmp2*$^{-/-}$) recipient (RCPT) mice. One month later, mice were infused or not with NE for 14 days. Mean 24-hour systolic blood pressure (SBP) was determined by telemetry (**A** and **B**). Values are means ± SEM. Number of samples per group for **A** = 3. For **B**: *Mmp2*$^{-/-}$→WT = 3 and *Mmp2*$^{-/-}$ →*Mmp2*$^{-/-}$ = 4.

Table S1. Antibodies for flow cytometry profiling of T cells

Antibodies	Description	Clone, company
CD3	A700-conjugated rat anti-mouse CD3 antibody	17A2, eBioscience
CD4	PerCP-e710-conjugated rat anti-mouse CD4 antibody	RM4-5, eBioscience
CD8a	APC-e780-conjugated rat anti-mouse-CD8a antibody	53-6.7, eBioscience
CD25	e450-conjugated rat anti-mouse-CD25 antibody	PC61.5, eBioscience
FOXP3	APC-conjugated rat anti-mouse-FOXP3 antibody	FJK-16s, eBioscience
CD69	PE-conjugated hamster anti-mouse CD69 antibody	H1.2F3, BD Biosciences

A700, Alexa Fluor® 700, APC, allophycocyanin, APC-e780, APC-eFluor® 780, e450, eFluor® 450, FOXP3, transcription factor X-linked forkhead/winged helix, PE, phycoerythrin, PerCP-e710, PerCP-eFluor® 710.

Table S2. Antibodies for flow cytometry profiling of monocytes

Antibodies	Description	Clone, company
CD90	PE-conjugated rat anti-mouse CD90.2 (Thy-1.2) antibody	53-2.1, eBioscience
B220	PE-conjugated rat anti-human/mouse CD45R (B220) antibody	RA3-6B2, eBioscience
CD49b	PE-conjugated anti-mouse CD49b (Integrin alpha 2) antibody	DX5, eBioscience
NK1.1	PE-conjugated mouse anti-mouse NK1.1 antibody	PK136, BD Biosciences
Ly-6G	PE-conjugated rat anti-Ly-6G (Gr-1) antibody	RB6-8C5, eBioscience
CD11b	APC-conjugated rat anti-mouse CD11b antibody	M1/70, eBioscience
Ly-6C	eFluor® 450 conjugated anti-mouse Ly-6C antibody	HK1.4, eBioscience

APC, allophycocyanin, PE, phycoerythrin. No antibody was used in replacement of Ly-6C isotype control antibody since this latter was not available.

Table S3. Oligonucleotides used in quantitative PCR assays

Gene	Forward	Reverse
Mmp2	5'-GGAGAAGGCTGTGTTCTTCG-3'	5'-TTTGGTTCTCCAGCTTCAGG-3'
Rps16	5'-ATCTCAAAGGCCCTGGTAGC-3'	5'-ACAAAGGTAAACCCCGATCC-3'

The ribosomal protein S16 (*Rps16*) gene was chosen as a reference gene for relative quantification.

Table S4. Oligonucleotides used in genotyping

Gene	Forward	Reverse
Mmp2	5'-CAACGATGGAGGCACGAGTG-3'	5'-GCCGGGGAACTTGATGATGG-3'
neo	5'-CTTGGGTGGAGAGGCTATTC-3'	5'-AGGTGAGATGACAGGAGATC-3'
Actb	5'-CGGTGCTAAGAAGGCTGTTC-3'	5'-ACCTGGGTCATCTTTTCACG-3'

The beta-actin (*Actb*) gene was chosen as a reference gene for relative quantification.