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Matrix metalloproteinase-2 knockout prevents angiotensin II-induced vascular injury

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Aims Matrix metalloproteinases (MMPs) have been implicated in the development of hypertension in animal models and humans. *Mmp2* deletion did not change Ang II-induced blood pressure (BP) rise. However, whether *Mmp2* knockout affects angiotensin (Ang) II-induced vascular injury has not been tested. We sought to determine whether *Mmp2* knockout will prevent Ang II-induced vascular injury.

Methods and results A fourteen-day Ang II infusion (1000 ng/kg/min, SC) increased systolic BP, decreased vasodilatory responses to acetylcholine, induced mesenteric artery (MA) hypertrophic remodelling, and enhanced MA stiffness in wild-type (WT) mice. Ang II enhanced aortic media and perivascular reactive oxygen species generation, aortic vascular cell adhesion molecule-1 and monocyte chemotactic protein-1 expression, perivascular monocyte/macrophage and T cell infiltration, and the fraction of spleen activated $CD4^{+}CD69^{+}$ and $CD8^{+}CD69^{+}$ T cells, and Ly-$6C^{hi}$ monocytes. Study of intracellular signalling showed that Ang II increased phosphorylation of epidermal growth factor receptor and extracellular-signal-regulated kinase 1/2 in vascular smooth muscle cells isolated from WT mice. All these effects were reduced or prevented by *Mmp2* knockout, except for systolic BP elevation. Ang II increased *Mmp2* expression in immune cells infiltrating the aorta and perivascular fat. Bone marrow (BM) transplantation experiments revealed that in absence of MMP2 in immune cells, Ang II-induced BP elevation was decreased, and that when MMP2 was deficient in either immune or vascular cells, Ang II-induced endothelial dysfunction was blunted.

Conclusions *Mmp2* knockout impaired Ang II-induced vascular injury but not BP elevation. BM transplantation revealed a role for immune cells in Ang II-induced BP elevation, and for both vascular and immune cell MMP2 in Ang II-induced endothelial dysfunction.

Keywords MMP2 • Blood pressure • Hypertension • Vascular injury • Bone marrow transplantation

1. Introduction

Vascular injury is an early manifestation leading to end-organ damage in hypertension.[1] Angiotensin (Ang) II, one of the final mediators of the renin–angiotensin–aldosterone system, plays an important role in the development of hypertension and vascular injury.[2] Ang II participates in the pathophysiology of hypertension by increasing blood pressure (BP) through vasoconstriction and its renal actions and by causing small artery injury. The latter is characterized by endothelial dysfunction and vascular remodelling, oxidative stress and inflammation.[1,3,4] However, the mechanisms whereby Ang II induces vascular injury are complex, and have not been definitively clarified in their entirety.

Matrix metalloproteinases (MMPs) have been implicated in the pathogenesis of hypertension and its complications.[5–8] MMPs are members of a superfamily of zinc-dependent endopeptidases that are involved in extracellular matrix (ECM) remodelling through collagen, elastin, and fibronectin degradation.[5,8–10] MMPs also play a role in vascular remodelling by shedding growth factors such as heparin-binding epidermal

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growth factor (HB-EGF)[11] and ECM-bound latent transforming growth factor (TGF) β.[12] In addition, MMPs could modulate vascular tone by cleaving big-endothelin-1 and other vasoactive peptides.[13] MMPs are expressed in the vascular system in vascular smooth muscle cells (VSMCs) and endothelial cells (ECs).[5] A systematic review and meta-analysis showed that plasma levels of MMP2, a gelatinase, are predictive of diastolic left ventricular dysfunction in hypertensive patients.[14] MMP2 expression is increased in cardiovascular tissues in hypertensive models such as Ang II-infused mice,[15,16] two-kidney, one-clip (2K-1C) rats,[17,18] and chronic nitric oxide (NO) synthase inhibition with N^{ω}-nitro-L-arginine methyl ester (L-NAME) in rats.[18] Transverse aortic constriction (TAC)-induced pressure overload also causes an elevation in cardiac MMP2 expression.[19] Interestingly, inhibition of MMPs with the non-selective MMP inhibitor doxycycline reduced the rise in BP and aortic endothelial dysfunction, hypertrophic remodelling and fibrosis in 2K-1C rats,[17] Doxycycline also blunted L-NAME-induced aortic hypertrophic remodelling but not mesenteric artery eutrophic remodelling in rats.[18] *Mmp2* targeted gene deletion prevented pressure overload-induced cardiac hypertrophy, dysfunction and fibrosis.[19] A MMP2 selective inhibitor or RNA interference targeting the *Mmp2* gene prevented Ang II-induced hypertension but not cardiac hypertrophy and fibrosis.[15] In another study, *Mmp2* knockout did not affect the development of hypertension but resulted in greater cardiac hypertrophy and fibrosis in Ang II-treated mice.[20] However, whether *Mmp2* knockout prevents the development of vascular injury in hypertension has never been tested.

We hypothesized that *Mmp2*-targeted gene deletion could prevent Ang II-induced vascular injury. In order to test this hypothesis, we first determined whether Ang II-induced hypertension and vascular injury were blunted in *Mmp2*$^{-/-}$ mice. Then we examined if Ang II signalling was reduced by *Mmp2* knockout in VSMCs *in vitro*. Finally, we determined the contribution of MMP2 expressed in vascular and immune cells to Ang II detrimental effects using bone marrow (BM) cell transplantation from wild-type (WT) into irradiated *Mmp2*$^{-/-}$ mice and vice versa.

2. Methods

An expanded Methods section is available in the Supplementary material online.

2.1 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 WT (Harlan laboratories, Indianapolis, IN, USA) and *Mmp2* knockout (*Mmp2*$^{-/-}$) mice (generously provided by Dr Shigeyoshi Itohara[21] and reproduced at the Lady Davis Institute for Medical Research) were anaesthetized with 3% isoflurane mixed with O_2 at 1 L/min. The depth of anaesthesia 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 mice were surgically implanted SC with ALZET osmotic mini pumps (Model 1002, Durect Corporation, Cupertino, CA, USA) infusing Ang II (1000 ng/kg/min) for 14 days, as previously described.[22] Control mice underwent sham surgery. A subset of the mice were anaesthetized with isoflurane and surgically instrumented with PA-C10 telemetry transmitters 7–10 days before Ang II treatment and BP was determined from two days before Ang II mini pump or sham surgery until the mice were sacrificed, as previously described.[22] At the end of the protocol, mice were weighed and then anaesthetized with isoflurane. The mesenteric arterial vascular bed was dissected, and tissues and tibia harvested in ice-cold phosphate buffered saline. Tissues were weighed and tibia length measured. The spleen was used for monocyte and T cell profiling. Second-order branches of mesenteric arteries (MA) were used for assessment of endothelial function and vessel mechanics by pressurized myography.[23] Portions of aorta were embedded in Clear Frozen Section Compound (VWR International, Edmonton, AL, Canada) for determination of reactive oxygen species (ROS) generation with the ROS-sensitive fluorescent dye dihydroethidium, expression of fibronectin, monocyte chemotactic protein-1 (MCP-1) and vascular cell adhesion molecule 1 (VCAM-1) or evaluation of tissue infiltration of monocyte/macrophages and T cells by immunofluorescence, or fixed with 4% paraformaldehyde and embedded in paraffin for quantification of collagen content with Sirius red staining.[24] The remaining tissues were frozen in liquid nitrogen and stored at -80 °C until used.

In order to examine the role of MMP2 in Ang II signalling in VSMCs, another set of 8–9-week-old male WT and *Mmp2*$^{-/-}$ mice were used to isolate the VSMCs from MA and study Ang II-induced epidermal growth factor receptor (EGFR) signalling.

In another subset of WT mice treated or not with Ang II as above, $CD45^{+}$ immune cells were isolated from thoracic aorta with the surrounding perivascular adipose tissue (PVAT) by fluorescence-activated cell sorting (FACS) and *Mmp2* expression was determined by reverse transcription (RT)-quantitative PCR (qPCR).

In order to elucidate the contribution of vascular tissue and BM-derived cell MMP2 to Ang II-induced hypertension and vascular injury, irradiation-BM transplantation experiments were performed using 8–10-week-old male WT and *Mmp2*$^{-/-}$ mice as BM donor and recipient. Ten million BM cells isolated from WT or *Mmp2*$^{-/-}$ mice were transplanted into γ-irradiated WT (WT → WT or *Mmp2*$^{-/-}$ → WT) or *Mmp2*$^{-/-}$ (WT → *Mmp2*$^{-/-}$ or *Mmp2*$^{-/-}$ → *Mmp2*$^{-/-}$) mice as previously described.[25] 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 two consecutive days before and during the 14-day period of treatment with or without Ang II and studied as above. In addition, blood was collected by cardiac puncture on EDTA. One hundred microlitre of blood was used for confirmation of BM reconstitution and the remaining blood was used for determination of plasma MMP2 and pro-MMP9. 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.[26]

2.2 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.

3. Results

3.1 BP, body and organ weights

Ang II infusion for 2 weeks caused a similar elevation in systolic BP in WT and *Mmp2*$^{-/-}$ mice (*Figure 1A*). Body weight, and heart, lung and kidney weight/tibia length were similar, whereas liver and spleen weight/tibia length were, respectively, smaller and greater in *Mmp2*$^{-/-}$ compared with WT mice (*Table 1*). Ang II induced an increase in the heart weight/tibia

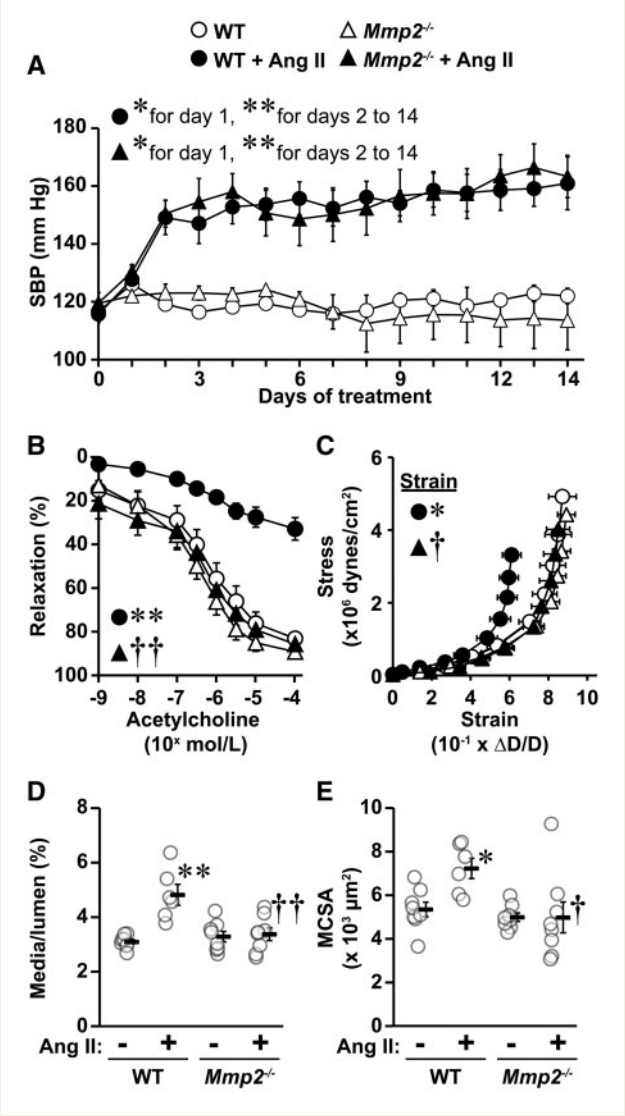

Figure 1 *Mmp2* gene deletion prevented angiotensin (Ang) II-induced endothelial dysfunction and vascular remodelling but not hypertension. Mean 24-h systolic blood pressure (SBP, *A*) by telemetry and vasodilator responses to acetylcholine (*B*), vascular stiffness (*C*), media/lumen (*D*), and media cross-sectional area (MCSA, *E*) of small mesenteric arteries using pressurized myography were determined in wild-type (WT) and *Mmp2* knockout (*Mmp2*$^{-/-}$) mice infused or not with Ang II for 14 days. Media/lumen and MCSA were determined at an intraluminal pressure of 45 mm Hg. Stiffness was determined by comparing values at 140 mm Hg. Values are means ± SEM. Number of samples per group for *A*: control groups = 3 and Ang II-treated groups = 5. For *B*: WT = 7, WT + Ang II = 6 and *Mmp2*$^{-/-}$ and *Mmp2*$^{-/-}$ + Ang II = 8. For *C–E*: WT + Ang II = 6 and other groups = 8. Data were analysed using two-way ANOVA for repeated measures in *A* and *B* and two-way ANOVA in *C–E*, with all ANOVA followed by a Student–Newman–Keuls *post hoc* test. Only the SBP of WT + Ang II and *Mmp2*$^{-/-}$ + Ang II were statistically analysed in *A* using respective days 0 as untreated controls. The SBP of WT and *Mmp2*$^{-/-}$ control groups is presented for reference, and is similar to day 0 of Ang II-treated WT and *Mmp2*$^{-/-}$ mice. The strain at 140 mm Hg was analysed in *C*. *$P < 0.05$ and **$P < 0.001$ vs. their respective controls and †$P < 0.05$ and ††$P < 0.001$ vs. WT + Ang II.

Table 1 Body and tissue weights

	WT	WT + Ang II	*Mmp2*$^{-/-}$	*Mmp2*$^{-/-}$ + Ang II
BW, g	27.6 ± 0.6	25.4 ± 0.5	27.1 ± 0.9	25.5 ± 0.8
HW/TL, mg/mm	7.5 ± 0.1	9.4 ± 0.4**	6.9 ± 0.3	8.4 ± 0.3*†
LuW/TL, mg/mm	10.2 ± 0.6	10.6 ± 0.5	10.8 ± 0.4	9.9 ± 0.9
KW/TL, mg/mm	10.9 ± 0.16	10.0 ± 0.3	11.0 ± 0.4	9.6 ± 0.4
LiW/TL, mg/mm	91.8 ± 1.6	88.8 ± 4.4	77.6 ± 4.4*	81.4 ± 5.5
SW/TL, mg/mm	3.7 ± 0.2	4.5 ± 0.4*	4.6 ± 0.1*	4.9 ± 0.2

Body weight (BW), tibia length (TL) and heart (HW), lung (LuW), kidney (KW), liver (LiW), and spleen (SW) weight were measured in wild-type (WT) and *Mmp2* knockout (*Mmp2*$^{-/-}$) mice infused or not with Ang II for 14 days. Values are mean ± SEM. Number of samples per group: *Mmp2*$^{-/-}$+Ang II = 8 and other groups = 9. Data were analyzed using two-way ANOVA followed by a Student-Newman-Keuls *post hoc* test.
*$P < 0.05$ vs. their respective WT or *Mmp2*$^{-/-}$ controls.
**$P < 0.001$ vs. their respective WT or *Mmp2*$^{-/-}$ controls.
†$P < 0.05$ vs. other Ang II-infused group.

length to a lesser extent in *Mmp2*$^{-/-}$ mice than WT mice. Ang II also caused an increase in spleen weight/tibia length in WT, but not in *Mmp2*$^{-/-}$ mice.

3.2 MMP2 is required for Ang II-induced endothelial dysfunction and vascular remodelling of small MA

Functional and mechanical properties of small MA were comparable in untreated WT and *Mmp2*$^{-/-}$ mice (*Figure 1B–E* and see Supplementary material online, *Figure S1*). Moreover, contractile responses to norepinephrine were unaffected by Ang II (see Supplementary material online, *Figure S1A*). Ang II caused impairment of the vasodilatory response to acetylcholine, as indicated by a 50% decrease in the maximal response, in WT but not in *Mmp2*$^{-/-}$ mice (*Figure 1B*). NO was the major mediator of the vasodilator response, since acetylcholine-induced relaxation was abrogated in all groups in the presence of the NO synthase inhibitor L-NAME (see Supplementary material online, *Figure 1B*). The impaired endothelial vasodilatory response was not due to a VSMC defect since endothelium-independent relaxation responses to the NO donor sodium nitroprusside were similar in all groups (see Supplementary material online, *Figure 1C*). In WT mice, Ang II also increased small artery stiffness as indicated by a leftward shift in the stress/strain curve (*Figure 1C*), and caused hypertrophic remodelling demonstrated by a ≥1.4-fold increase in the media/lumen and cross-sectional area of mesenteric resistance arteries (*Figure 1D* and *E*). *Mmp2* deletion prevented Ang II-induced endothelium-dependent relaxation response dysfunction, increase in small artery stiffness and hypertrophic remodelling.

3.3 MMP2 is required for Ang II-induced vascular ROS generation and ECM remodelling

Ang II increased ROS generation 10-fold in the aortic media and six-fold in the adventitia and PVAT in WT mice (*Figure 2A* and D). ROS generation was reduced in *Mmp2*$^{-/-}$ mice. Ang II-treated WT mice presented a 1.5-fold increase in the expression of aortic media fibronectin (*Figure 2B* and *E*) and a 1.7-fold increase in adventitial collagen fraction (*Figure 2C* and *F*) compared with untreated WT mice, changes not found in *Mmp2*$^{-/-}$ mice.

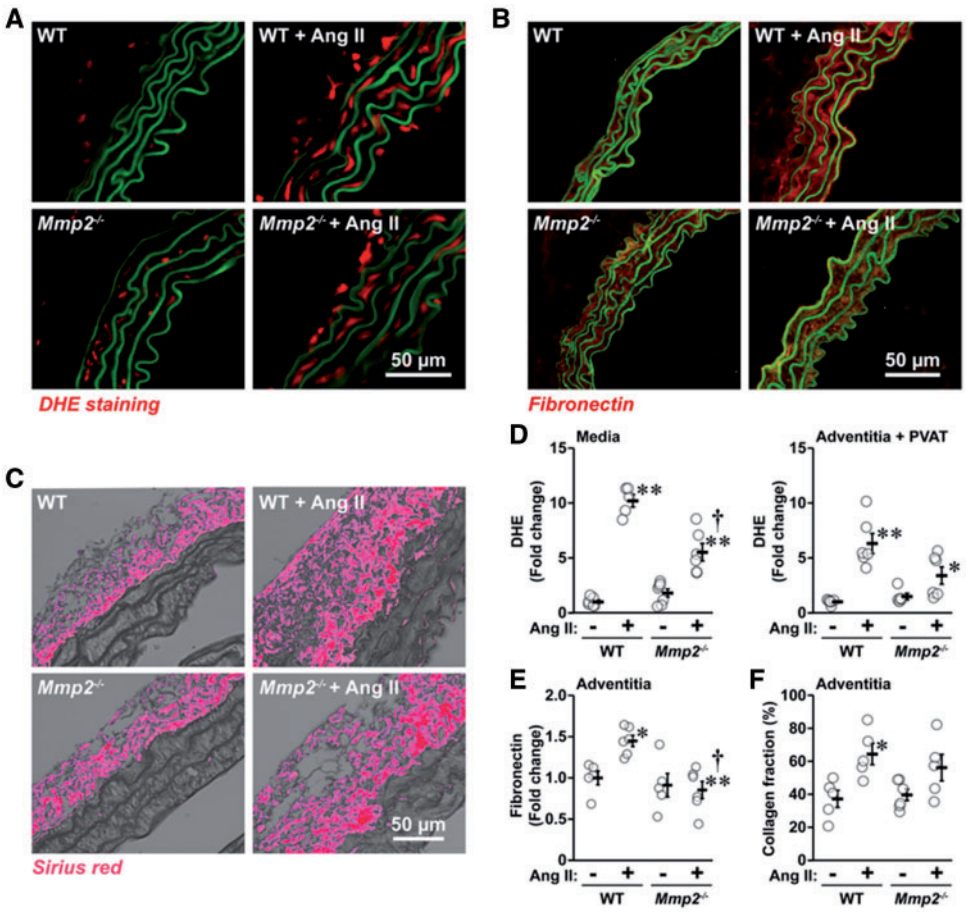

Figure 2 *Mmp2* gene deletion reduced Ang II-induced reactive oxygen species (ROS) generation and extracellular matrix remodelling. ROS generation by dihydroethidium (DHE) staining in the aortic media and adventitia and perivascular adipose tissue (PVAT) (*A* and *D*), media fibronectin expression by immunofluorescence (*B* and *E*) and adventitial collagen content by Sirius red staining (*C* and *F*) were determined in the same groups as in *Figure 1*. Representative images of DHE staining (*A*), fibronectin (*B*) immunofluorescence images and RGB thresholded images of Sirius red staining (*C*) of aortic sections are shown. Green fluorescence in *A* and *B* represents elastin autofluorescence. Values are means ± SEM. Number of samples per group for media in *D*: $Mmp2^{-/-}$ = 7 and other groups = 6. For Adventitia + PVAT in *D*: $Mmp2^{-/-}$ = 5 and other groups = 6. For *E*: controls = 5 and Ang II-treated groups = 6. For *F*: $Mmp2^{-/-}$ + Ang II = 6 and other groups = 5. Data were analysed using two-way ANOVA followed by a Student–Newman–Keuls *post hoc* test. **P* < 0.05 and ***P* < 0.001 vs. their respective controls and †*P* < 0.001 vs. WT + Ang II.

3.4 MMP2 is required for Ang II-induced vascular inflammation and immune response

Ang II increased aortic VCAM-1 and MCP-1 expression four- and seven-fold, respectively, in WT mice, increases that were reduced in $Mmp2^{-/-}$ mice (*Figure 3A, B* and *E, F*). Ang II caused an elevation in monocyte/macrophage (15-fold) and $CD3^+$ T cell (four-fold) infiltration in PVAT of WT mice, effect that was markedly reduced in $Mmp2^{-/-}$ mice (*Figure 3C, D* and *G, H*).

MMP2 is required for activation of immune cells at a systemic level. This was demonstrated by the finding that Ang II caused a 50% decrease in the fraction of $CD11b^+$ monocytes and a two-fold increase in activated Ly-$6C^{hi}$ monocytes in the spleen of WT mice, but not in $Mmp2^{-/-}$ mice (*Figure 4A* and *B*). It should be noted that the fraction of $CD11b^+$ monocytes was lower in $Mmp2^{-/-}$ than in WT mice and not affected by Ang II treatment. The fraction of pan ($CD3^+$) T cells, T helper ($CD3^+CD4^+$) cells, cytotoxic ($CD3^+CD8^+$) T cells, and T regulatory cells (Treg, $CD4^+CD25^+FOXP3^+$) in the spleen of WT mice was unaffected by Ang II treatment (see Supplementary material online, *Figure S2*). The fraction of pan T cells was lower in the spleen of $Mmp2^{-/-}$ mice, and Ang II increased this fraction to a comparable level to that observed in WT mice. The fraction of T helper cells, cytotoxic T cells, and Treg was unaltered by deficiency of MMP2 or by Ang II treatment. Ang II increased the fraction of activated T helper ($CD4^+CD69^+$) and cytotoxic ($CD8^+CD69^+$) T cells ≥two-fold in the spleen of WT mice, but not in $Mmp2^{-/-}$ mice (*Figure 4C* and *D*).

3.5 MMP2 is required for Ang II-EGFR-dependent signalling in VSMCs

The function of MMPs is not limited to turnover and degradation of ECM, but also involves shedding of HB-EGF with subsequent activation of EGFR and downstream mitogen-activated protein kinases (MAPKs), which could be involved in Ang II-induced vascular injury.[27] Since MMP2 is highly expressed in VSMCs, we investigated whether *Mmp2* deficiency affects Ang II signalling in VSMCs isolated from MA in order to demonstrate the

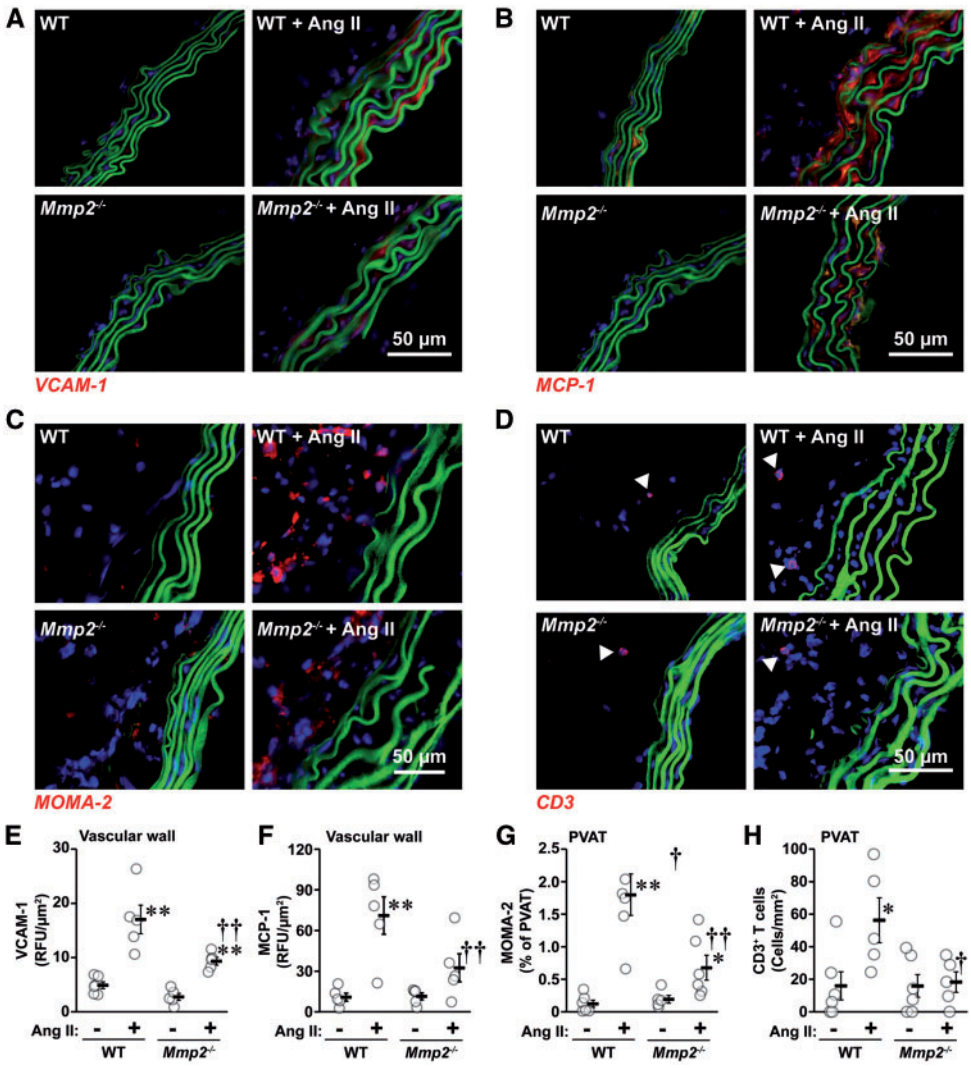

Figure 3 *Mmp2* gene deletion reduced Ang II-induced inflammation. Aortic VCAM-1 (*A* and *E*) and MCP-1 (*B* and *F*) expression and MOMA-2$^+$ monocyte/macrophage (*C* and *G*) and CD3$^+$ T cell (*D* and *H*) infiltration were determined by immunofluorescence in the same groups as in *Figure 1*. Representative VCAM-1 (*A*, in red), MCP-1 (*B*, in red), MOMA-2$^+$ monocyte/macrophages (*C*, in red), and CD3$^+$ T cell (*D*, in red) immunofluorescence images of aortic sections are shown. Elastin autofluorescence and nuclear stain DAPI are shown in green and blue, respectively. Arrow heads indicate CD3$^+$ T cells in *D*. Values are means ± SEM. Number of samples per group for *E*: WT = 6 and other groups = 5. For *F*: all groups = 5. For *G*: *Mmp2*$^{-/-}$ = 5 and other groups = 6. For *H*: WT and *Mmp2*$^{-/-}$ = 5 and Ang II-treated groups = 6. Data were analysed using two-way ANOVA followed by a Student–Newman–Keuls *post hoc* test. *$P < 0.05$ and **$P < 0.001$ vs. their respective untreated controls and $^{\dagger}P < 0.05$ and vs. $^{\dagger\dagger}P < 0.01$ WT + Ang II.

mechanism whereby MMP2 deficiency results in the effects demonstrated in this study. Ang II-induced phosphorylation of EGFR and the downstream extracellular-signal-regulated kinases 1/2 (ERK1/2), MAPKs, were therefore examined in VSMCs. Ang II increased phosphorylation of EGFR two-fold and ERK1/2 1.4-fold in VSMCs isolated from MA of WT (*Figure 5*). These effects were absent in VSMCs of *Mmp2*$^{-/-}$ mouse, indicating that MMP2 acts by mediating MAPK activation via EGFR phosphorylation, presumably through contributing to the shedding of HB-EGF.[11]

3.6 Ang II induced *Mmp2* expression in infiltrating immune cells

Since MMP2 is expressed in peripheral blood monocytes, resident macrophages and T lymphocytes,[9,28,29] we investigated whether Ang II treatment increased the expression of *Mmp2* in immune cells infiltrating the aorta/PVAT in WT mice. Ang II increased seven-fold the infiltration of pan (CD45$^+$) immune cells in the aorta/PVAT (*Figure 6A*), and caused a three-fold rise in *Mmp2* expression in these cells (*Figure 6B*).

3.7 Relative role of immune and vascular cell MMP2 in development of Ang II-induced hypertension and endothelial dysfunction

Since MMP2 expressed in immune cells has been implicated in atherosclerotic plaque rupture[28,29] and in Ang II-induced hypertension and vascular injury,[3,4] we investigated using BM cell transplantation whether MMP2 expressed in vascular tissue, immune cells, or both, contributed

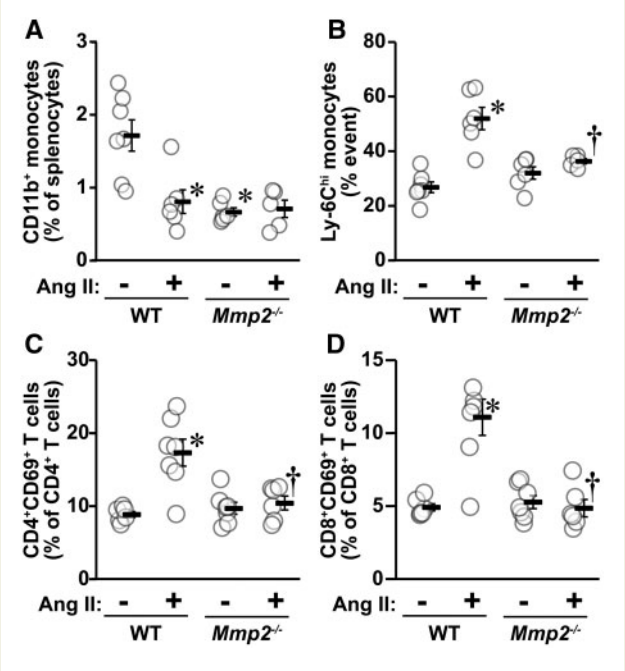

Figure 4 *Mmp2* gene deletion blunted Ang II-induced immune responses. Spleen CD11b^{+} monocytes (*A*), activated Ly-6C^{hi} monocytes (*B*), CD4^{+}CD69^{+} T cells (*C*), and CD8^{+}CD69^{+} T cells (*D*) were determined by flow cytometry in the same groups as in *Figure 1*. Values are means ± SEM. Number of samples per group for *A* and *B*: WT = 7, WT + Ang II and *Mmp2*$^{-/-}$ = 6 and *Mmp2*$^{-/-}$ + Ang II = 5. For *C* and *D*: WT and *Mmp2*$^{-/-}$ + Ang II = 6 and other groups = 7. Data were analysed using two-way ANOVA followed by a Student–Newman–Keuls *post hoc* test. **P* < 0.001 vs. their respective controls and $^{\dagger}P < 0.01$ and $^{\dagger\dagger}P < 0.001$ vs. WT + Ang II.

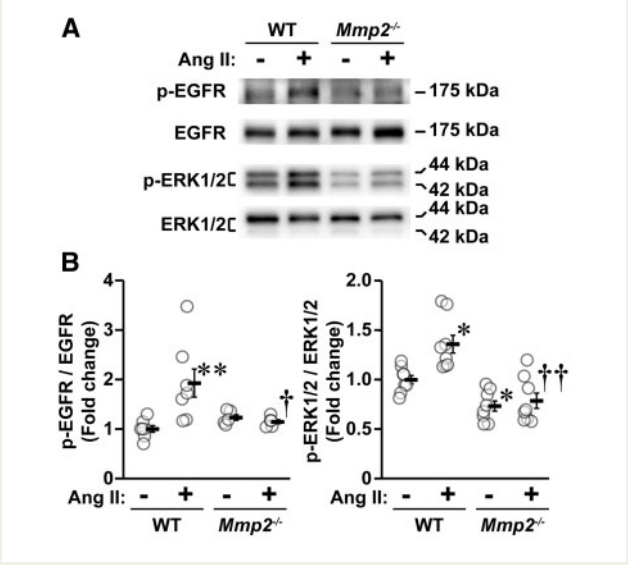

Figure 5 *Mmp2* gene deletion blunted Ang II-induced signalling in vascular smooth muscle cells (VSMCs). The level of phosphorylation of epidermal growth factor receptor (EGFR) and of p44/42 mitogen-activated protein kinase (extracellular-signal-regulated kinase 1/2, ERK1/2) in VSMCs from small mesenteric arteries cultured in presence or absence of Ang II for 5 min were determined by western blot. Representative western blots of phosphorylated (p) and total EGFR and ERK1/2 (*A*) and corresponding dot plots (*B*) are represented. Values are means ± SEM, number of sample per group for p-EGFR/EGFR: WT = 6, *Mmp2*$^{-/-}$ = 7 and other groups = 5, and for p-ERK1/2/ERK1/2: eight per group. Data were analysed using two-way ANOVA followed by a Student–Newman–Keuls *post hoc* test. **P* < 0.01 and ***P* < 0.001 and vs. their respective controls and $^{\dagger}P < 0.01$ and vs. $^{\dagger\dagger}P < 0.01$ WT + Ang II.

to Ang II-induced BP elevation, endothelial dysfunction and vascular remodelling. WT and *Mmp2*$^{-/-}$ mice were irradiated and transplanted with BM cells from WT or *Mmp2*$^{-/-}$ mice, and vice versa. Successful BM cell reconstitution was demonstrated by quantitative PCR of *Mmp2* gene (WT donor marker) and neomycin (*neo*) resistance gene (*Mmp2* KO donor marker) in whole blood (see Supplementary material online, *Figure S3*).

To further characterize the results of BM transplantation mice, plasma MMP2 was determined. MMP2 was detected in the plasma of WT mice transplanted with WT or *Mmp2*$^{-/-}$ BM cells (WT → WT and *Mmp2*$^{-/-}$ → WT), and barely or not at all in *Mmp2*$^{-/-}$ mice transplanted with WT or *Mmp2*$^{-/-}$ BM cells (WT → *Mmp2*$^{-/-}$and *Mmp2*$^{-/-}$ → *Mmp2*$^{-/-}$; see Supplementary material online, *Figure S4A* and *B*). Ang II infusion caused a 3.6-fold elevation in plasma MMP2 in WT → WT mice. *Mmp2*$^{-/-}$ → WT mice presented 2.3-fold more plasma MMP2 than WT → WT mice, which was not further altered by Ang II infusion. In order to determine whether lack of MMP2 could be compensated by another MMP, plasma pro-MMP9 was measured. Plasma pro-MMP9 levels were similar in WT → WT, *Mmp2*$^{-/-}$ → WT and WT → *Mmp2*$^{-/-}$ mice, and were unaffected by Ang II infusion (see Supplementary material online, *Figure S4C* and *D*). However, *Mmp2*$^{-/-}$ → *Mmp2*$^{-/-}$ mice presented 36% lower plasma pro-MMP9 compared with WT → *Mmp2*$^{-/-}$ mice, which upon Ang II infusion increased to a level similar to that observed in the other groups.

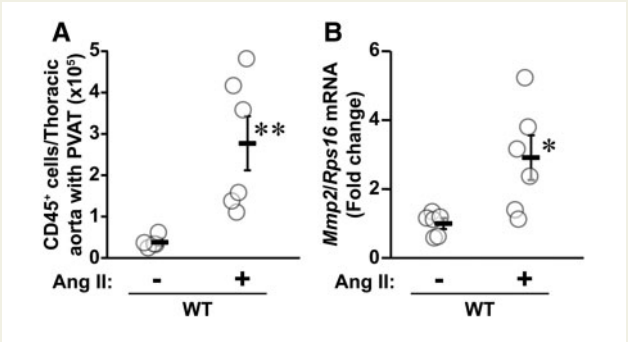

Figure 6 Angiotensin (Ang) II caused an increase in *Mmp2* mRNA expression in aorta/perivascular adipose tissue (PVAT) infiltrating pan (CD45^{+}) immune cells. The infiltrating CD45^{+} immune cells isolated from aorta/perivascular adipose tissue (PVAT) by fluorescence-activated cell sorting and the mRNA expression of *Mmp2* and ribosomal protein S16 (*Rps16*) assessed by reverse transcription-quantitative PCR were determined in wild-type (WT) mice infused or not with Ang II for 14 days. Values are means ± SEM, number of sample per group: WT = 5 and WT + Ang II = 6. Data were analysed using an unpaired *T* test. **P* < 0.05 and ***P* < 0.001 and vs. WT controls.

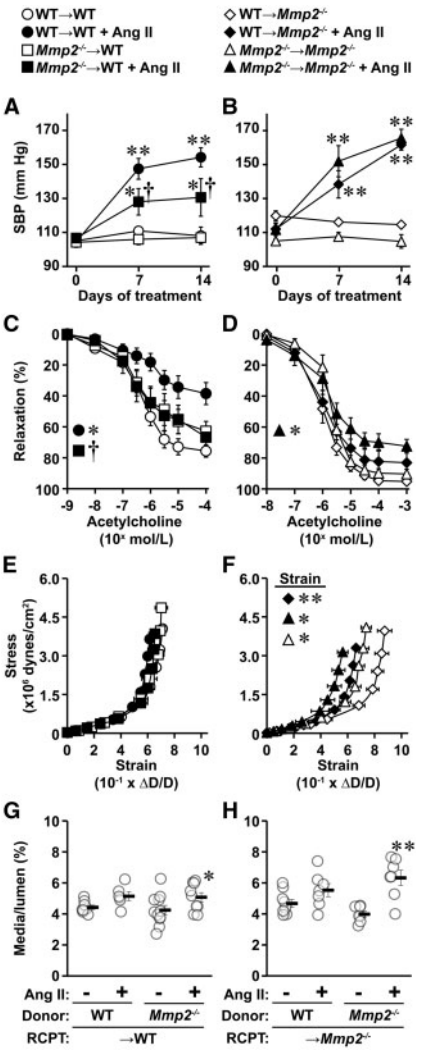

Figure 7 Absence of MMP2 in immune cells decreased Ang II-induced rise in SBP, and lack of MMP2 in immune or vascular cells blunted Ang II-induced endothelial dysfunction. Bone marrow from wild-type (WT) and *Mmp2*$^{-/-}$ donor mice was transplanted into γ-irradiated WT (*A*, *C*, *E* and *G*, WT → WT and *Mmp2*$^{-/-}$ → WT) and *Mmp2*$^{-/-}$ (*B*, *D*, *F* and *H*, WT → *Mmp2*$^{-/-}$ and *Mmp2*$^{-/-}$ → *Mmp2*$^{-/-}$) recipient (RCPT) mice. One month later, mice were infused or not with Ang II for 14 days. Mean 24-h SBP was determined by telemetry (*A* and *B*). Vasodilator responses to acetylcholine (*C* and *D*), vascular stiffness (*E* and *F*), and remodelling (*G* and *H*) of small mesenteric arteries were determined by pressurized myography at the end of the infusion period. Media/lumen was determined with an intraluminal pressure of 45 mm Hg. Values are means ± SEM. Number of samples per group for *A*: WT → WT and *Mmp2*$^{-/-}$ → WT + Ang II = 6 and other groups = 7. For *B*: WT → *Mmp2*$^{-/-}$ + Ang II = 6 and other groups = 5. For *C*: WT → WT = 8, WT → WT + Ang II = 7, *Mmp2*$^{-/-}$ → WT = 9, and *Mmp2*$^{-/-}$ → WT + Ang II = 10. For *D*: WT → *Mmp2*$^{-/-}$ = 8, *Mmp2*$^{-/-}$ → *Mmp2*$^{-/-}$ = 7, and other groups = 6. For *E* and *G*: WT → WT = 8, WT → WT + Ang II = 6, *Mmp2*$^{-/-}$ → WT = 11, and *Mmp2*$^{-/-}$ → WT + Ang II = 9. For *F* and *H*: WT → *Mmp2*$^{-/-}$ = 9 and other groups = 7. Data were analysed using two-way ANOVA for repeated measures in *A*–*D* and two-way ANOVA in *E*–*H*, with all ANOVA followed by a Student–Newman–Keuls *post hoc* test. SBP at days 7 and 14 (*A* and *B*) and vasodilator responses to acetylcholine 10^{-6} to 10^{-4} mol/L (*C* and *D*) were statically analysed. The strain at 140 mm Hg was analysed in *F*. **P* < 0.05 and ***P* < 0.001 vs. their respective controls, and $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.001$ vs. other Ang II-treated group.

Fourteen days of Ang II caused a 46 and 61 mm Hg mean rise in systolic BP in WT → WT and *Mmp2*$^{-/-}$ → *Mmp2*$^{-/-}$ mice, respectively (*Figure* 7*A* and *B*). The systolic BP was 24 mm Hg lower in *Mmp2*$^{-/-}$ → WT mice than WT → WT mice, and similar in WT → *Mmp2*$^{-/-}$ compared with *Mmp2*$^{-/-}$ → *Mmp2*$^{-/-}$ mice. After Ang II treatment, the MA vasodilatory response to acetylcholine was reduced by ∼50% in WT → WT and to a lesser extent (20%) in *Mmp2*$^{-/-}$ → *Mmp2*$^{-/-}$, but was not blunted in either in *Mmp2*$^{-/-}$ → WT or WT → *Mmp2*$^{-/-}$ mice (*Figure* 7*C* and *D*). Small MA stiffness of WT → WT and *Mmp2*$^{-/-}$ → WT mice was unaffected by Ang II treatment, whereas it was increased in both WT → *Mmp2*$^{-/-}$ and *Mmp2*$^{-/-}$ → *Mmp2*$^{-/-}$ mice, as indicated by a leftward shift in the stress/strain curves (*Figure* 7*E* and *F*). It should be noted that control *Mmp2*$^{-/-}$ → *Mmp2*$^{-/-}$ mice presented stiffer MA than control WT → *Mmp2*$^{-/-}$ mice (Figures 7F). Finally, Ang II caused vascular remodelling in small MA in *Mmp2*$^{-/-}$ → WT and *Mmp2*$^{-/-}$ → *Mmp2*$^{-/-}$ mice, demonstrated by a 1.2- and 1.6-fold increase in media/lumen, respectively, but not in the other groups (Figure 7G and *H*). Media cross-sectional area was smaller in *Mmp2*$^{-/-}$ → *Mmp2*$^{-/-}$ compared with WT → *Mmp2*$^{-/-}$ mice, and increased 1.6-fold by Ang II infusion in *Mmp2*$^{-/-}$ → *Mmp2*$^{-/-}$, and was unaffected in the other groups (see Supplementary material online, *Figure S5*).

ROS generation was similar in the aortic media of all BM transplanted mice (see Supplementary material online, *Figure* S6A–D). However, ROS generation was increased in adventitia and PVAT upon Ang II infusion in WT → WT, WT → *Mmp2*$^{-/-}$ and *Mmp2*$^{-/-}$ → *Mmp2*$^{-/-}$ mice but not in *Mmp2*$^{-/-}$ → WT mice (see Supplementary material online, *Figure S6A, B*, and *E, F*). Control *Mmp2*$^{-/-}$ → *Mmp2*$^{-/-}$ mice presented lower ROS generation than control WT → *Mmp2*$^{-/-}$ mice (see Supplementary material online, *Figure S6B* and *F*).

In order to determine whether the alteration in Ang II-induced BP rise in absence of vascular or immune MMP2 was selective to this model, an alternative hypertensive agent was tested. Norepinephrine infusion caused similar SBP rise in all groups (see Supplementary material online, *Figure S7*).

4. Discussion

The present study showed that *Mmp2* deletion prevents Ang II-induced endothelial dysfunction, vascular remodelling, oxidative stress, and inflammation in mice. These vascular protective effects could be mediated at least in part through the inhibition of the role that MMP2 plays via shedding of HB-EGF[11] leading to EGFR/ERK1/2 signalling in VSMCs. Ang II increased *Mmp2* expression in aorta/PVAT infiltrating immune cells. As well, BM cell transplantation experiments revealed that *Mmp2* deficiency in immune cells reduced BP rise, and lack of *Mmp2* in both vascular and immune cells blunted endothelial dysfunction in Ang II infused mice.

In this study, endothelial dysfunction, vascular stiffness and remodelling of small MA, oxidative stress and inflammation caused by Ang II were blunted by *Mmp2* deletion, whereas BP elevation was not. This is the first study that demonstrates a role of MMP2 in the development of vascular injury independent of BP elevation. The role of MMPs in hypertension has been examined previously using doxycycline, a broad-spectrum MMP inhibitor. Bouvet et al. showed that doxycycline reduced BP elevation and aortic remodelling but not mesenteric artery remodelling in rats subjected to chronic NO synthase inhibition with L-NAME.[18] In 2K-1C hypertension, Castro et al. observed that doxycycline reduced BP and prevented development of aortic endothelial dysfunction and

remodelling.[17] The blunting of BP elevation in the above studies could be due to inhibition of multiple MMPs and also to off-target effects of the non-selective MMP inhibitors. Odenbach et al. showed that specific pharmacologic inhibition of MMP2 using the lipid analogue MMP2 inhibitor I, and RNA interference to knock down *Mmp2*, prevented Ang II-induced hypertension but not cardiac hypertrophy and fibrosis.[15] More recently, the same group showed that the extent of BP elevation was unaffected but cardiac hypertrophy and pericoronary artery fibrosis were exaggerated in Ang II-treated *Mmp2*$^{-/-}$ compared with WT mice.[20] This latter study is in agreement with our current study with respect to BP. However, in our study cardiac hypertrophy was unaffected by absence of MMP2.

MMP2 is expressed in different tissues including vascular cells. It is highly expressed in VSMCs as demonstrated by *in silico* human expression profile (http://ds.biogps.org/?dataset=GSE1133&gene=4313, accessed on 7 May 2017, date last accessed). Furthermore, Ang II increased expression of *Mmp2 in vitro* in VSMCs and *in vivo* in mouse aorta.[16,30–33] Therefore, it is possible that prevention of Ang II-induced vascular injury by *Mmp2* knockout could be due, at least in part, to the lack of MMP2 in VSMCs. MMPs including MMP2 have been involved in activation or shedding of growth factors and peptides such as latent TGFβ,[34] big endothelin-1,[13] and HB-EGF.[27] The mitogenic effects of the G protein-coupled receptors, including Ang type 1 receptor (AGTR1), are mediated through activation of EGFR and subsequent activation of phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and MAPKs including ERK1/2 and p38 MAPK.[11,27] Indeed, our study of cultured mesenteric artery VSMCs demonstrated that Ang II signalling via EGFR and ERK1/2 was blunted in absence of MMP2. Also, Schreier et al. showed that postnatal VSMC *Egfr* knockout blunted acute vasoconstriction induced by Ang II.[35] Accordingly, vascular protective effects of *Mmp2* knockout could be mediated at least in part by inhibition of Ang II signalling in VSMCs. A similar protective mechanism may be occurring in other cell types such as immune cells. Indeed, monocyte/macrophages have been shown to express AGTR1,[36] *Mmp2*,[28,29] and EGFR.[37,38]

We and others have shown that the innate and adaptive immune systems play an important role in the development of hypertension and vascular injury.[3,4] PVAT infiltrating immune cells could cause endothelial dysfunction through ROS generation. MMP2 expression is induced in monocytes via interaction with ECM components such as fibronectin, while it is constitutively expressed in differentiated macrophages.[28] MMP2 is expressed to a higher level in CD4^{+} T helper (T_h)1 effector cells than in T_h2 effector or naive T_h0 cells. Furthermore, T_h1 effector cells can stimulate MMP2 expression in macrophages.[29] In this study, we demonstrated for the first time that Ang II increased *Mmp2* expression in aorta/PVAT infiltrating immune cells, which could contribute to Ang II-induced vascular injury. This possibility was addressed using BM cell transplantation from WT or *Mmp2*$^{-/-}$ mice into WT or *Mmp2*$^{-/-}$ mice.

BM cell transplantation experiments demonstrated a role for immune cell MMP2 in the development of hypertension. Ang II-induced BP rise was reduced in WT recipient mice that received *Mmp2*$^{-/-}$ BM cells, and was delayed in *Mmp2*$^{-/-}$ recipient mice that received WT or *Mmp2*$^{-/-}$ BM cells. This finding might be unique to the Ang II-infused model since it was not observed using BM-transplanted mice infused with norepinephrine as an alternative hypertensive agent. It is unclear why BP did not reach the same levels after Ang II infusion in WT and *Mmp2*$^{-/-}$ recipient mice. However, this difference was not observed with norepinephrine infusion.

A role for both immune and vascular cell MMP2 was also demonstrated for mesenteric artery endothelial function. The most severe endothelial dysfunction caused by Ang II was observed in mice having MMP2 in both immune and vascular cells, and deletion of *Mmp2* in either immune cells, vascular cells or both blunted or prevented Ang II-induced endothelial dysfunction. Ang II-induced endothelial dysfunction is mediated at least in part by increased production of oxidative stress by immune cells.[22,39] Deletion of MMP2 in immune cells accompanied by persistence of vascular MMP2 was associated with blunted Ang II-induced perivascular ROS production. However, a dissociation between endothelial function and perivascular ROS generation was observed in *Mmp2*$^{-/-}$ recipient mice receiving either WT or *Mmp2*$^{-/-}$ BM cells.

The Ang II-induced increase in vascular stiffness was found only in *Mmp2*$^{-/-}$ recipient mice receiving WT or *Mmp2*$^{-/-}$ BM cells, whereas Ang II-induced vascular remodelling was observed only in WT and *Mmp2*$^{-/-}$ recipient mice receiving *Mmp2*$^{-/-}$ BM cells. The slightly greater increase in BP observed in *Mmp2*$^{-/-}$ recipient mice receiving WT or *Mmp2*$^{-/-}$ BM cells compared with WT mice receiving either BM cells could have resulted in the observed vascular stiffening. Differences in vascular wall composition (perhaps due to the irradiation necessary for BM transplantation) could explain the vascular stiffening and remodelling results. As a consequence, BM cell transplantation experiments did not totally reproduce the experiments performed originally with WT and *Mmp2*$^{-/-}$ mice infused with Ang II. However, they allowed teasing out differential effects of MMP2 expressed in either immune cells or in vascular tissue. The limitations of BM cell transplantation experiments could only be resolved by using an inducible conditional gene knockout mouse. Unfortunately, floxed *Mmp2* mice are unavailable.

There are similarities between these results and our previous findings showing that Ang II-induced BP rise and endothelial dysfunction were reduced or blunted in osteopetrotic mice that have a mutation in the colony-stimulating factor gene (*Csf1*) and an associated decrease in functional monocyte/macrophages,[40] as well as in mice adoptively transferred with T regulatory cells,[22] in which effector T cells and innate immune cells such as dendritic cells, monocytes, and macrophages are suppressed.[3,4] In this study, *Mmp2* deficiency in immune cells prevented Ang II-induced BP rise and endothelial dysfunction. MMP2 could play a role in innate or adaptive immune responses in the pathophysiology of hypertension and vascular injury. This could be mediated through a local MMP2 action, since immune cells contribute very little to circulating MMP2. It has been shown that interaction of monocytes with matrix leads to up-regulation of several MMPs including MMP2.[28] Interestingly, we found that Ang II enhanced MMP2 expression in infiltrating immune cells. MMP2 could contribute to immune cell activation via cytokines such as interleukin-1β.[9] We also observed that the lack of *Mmp2* in vascular tissue did not affect Ang II-induced BP rise but blunted endothelial dysfunction. It is unclear why the lack of vascular MMP2 did not impede Ang II pressor responses. However, vascular MMP2 might be important for Ang II-induced vascular injury. Expression of MMP2 in vascular cells could play a role in the attraction and recruitment of monocytes and T cells, as has been suggested in atherosclerosis.[28] This could occur via Ang II-dependent MMP2-mediated EGFR signalling by vascular cells or infiltrating monocyte/macrophages expressing AGTR1, MMP2, and EGFR.[22,41] This could also rely on MMP2 secretion by vascular or immune cells causing degradation of ECM or activation of nearby immune cells.

5. Limitations

The present study has focused on determining the role of MMP2 in Ang II-induced hypertension and vascular injury. MMP2 is part of the MMP

family that includes 24 members.[9] MMPs are regulated by tissue inhibitors of metalloproteinases (TIMPs) that inhibit their activity by binding to their catalytic site. There are four TIMPs (TIMP-1, -2, -3 and -4). TIMP2 acts as inhibitor or activator of MMPs. TIMP-2 is required with MMP14 for the activation of pro-MMP2. The present study has not addressed the role of the complex interactional network of MMPs/TIMPS in hypertension and vascular injury, for which additional studies will be required.

6. Conclusions and perspectives

This study demonstrated using *Mmp2* null mice that MMP2 mediates effects of Ang II leading to endothelial dysfunction, vascular remodelling, oxidative stress, and inflammation. These effects are mediated at least in part through the role that MMP2 plays via shedding of HB-EGF that stimulate EGFR/ERK1/2 signalling in VSMCs, or through enhanced *Mmp2* expression in infiltrating immune cells. BM transplantation experimental results revealed a role for immune cell MMP2 in Ang II-induced BP elevation and for both vascular and immune cell MMP2 in Ang II-induced endothelial dysfunction. Understanding the mechanism of action of MMP2 in immune and vascular cells may reveal new therapeutic targets for the treatment of hypertension and vascular disease.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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