

# Critical Role of Monocyte Chemoattractant Protein-1 Receptor CCR2 on Monocytes in Hypertension-Induced Vascular Inflammation and Remodeling

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**Abstract**—Activated monocytes are present in the arterial walls of hypertensive patients and animals. Monocyte chemoattractant protein-1 (MCP-1), which controls monocyte function through its receptor (CCR2), is implicated in hypertensive inflammatory changes in the arterial wall. The role of CCR2 expression on monocytes in hypertension-induced vascular remodeling, however, has not been addressed. We hypothesized that CCR2 on monocytes is critical in hypertension-induced vascular inflammation and remodeling. Hypertension was induced by infusion of angiotensin II (Ang II) into wild-type mice, CCR2-deficient (CCR2<sup>-/-</sup>) mice, and bone marrow-transferred mice with a leukocyte-selective CCR2 deficiency (BMT-CCR2<sup>-/-</sup>). In wild-type mice, Ang II increased CCR2 intensity in circulating monocytes, which was prevented by an Ang II type-1 (AT<sub>1</sub>) receptor blocker or blunted in AT<sub>1</sub> receptor-deficient mice. Enhanced CCR2 intensity on monocytes was observed in hypertensive patients and rats, and was reduced by treatment with the Ang II receptor blocker, supporting the clinical relevance of the observation in mice. In CCR2<sup>-/-</sup> and BMT-CCR2<sup>-/-</sup> mice, Ang II-induced vascular inflammation and vascular remodeling (aortic wall thickening and fibrosis) were blunted as compared with control mice. In contrast, Ang II-induced left ventricular hypertrophy developed in CCR2<sup>-/-</sup> and BMT-CCR2<sup>-/-</sup> mice. The present study suggests that CCR2 expression in monocytes has a critical role in vascular inflammation and remodeling in Ang II-induced hypertension, and possibly in other forms of hypertension. (*Circ Res.* 2004;94:1203-1210.)

**Key Words:** vascular remodeling ■ angiotensin II ■ inflammation ■ leukocytes

Chronic monocyte-mediated inflammation in arterial walls is observed in hypertensive patients and animals.<sup>1-3</sup> Recent clinical studies reported that lowering angiotensin II (Ang II) activity is a practical target of therapy for patients with cardiovascular disease.<sup>4-6</sup> Ang II mediates reactive oxidative species (ROS) and stimulates the release of cytokines and growth factors (interleukin-6) and the expression of adhesion molecules (vascular cell adhesion molecule-1) and chemokines [monocyte chemoattractant protein-1 (MCP-1)] that mediate arterial wall inflammation.<sup>1-3</sup> For example, Ang II can induce monocyte chemotaxis by producing MCP-1 from vascular smooth muscle cells and monocytes through NF- $\kappa$ B.<sup>7,8</sup>

MCP-1 is a C-C chemokine that controls monocyte recruitment to the site of inflammation through its receptor, C-C chemokine receptor (CCR) 2.<sup>9-11</sup> The MCP-1/CCR2 pathway appears to be involved in the inflammatory aspect of hyper-

tensive artery disease. MCP-1 and CCR2 expression and activity are enhanced in the arterial walls of hypertensive animals.<sup>12,13</sup> Furthermore, activation of the MCP-1/CCR2 pathway induces monocyte-mediated inflammation, as well as production of adhesion molecules,<sup>14</sup> inflammatory cytokines,<sup>15</sup> and tissue factor,<sup>16</sup> and stimulates migration of vascular smooth muscle cells, resulting in neointimal hyperplasia or atherosclerosis.<sup>17-20</sup> We previously demonstrated that blockade of the MCP-1/CCR2 pathway prevents vascular inflammation and arteriosclerosis in rats made hypertensive by chronic inhibition of nitric oxide synthesis.<sup>13,21-24</sup> Blockade or abrogation of MCP-1 or CCR2 markedly attenuates the early development of atherosclerosis as well as the progression and destabilization of established lesions in hyperlipidemic mice.<sup>25-28,29</sup> CCR2-deficient (CCR2<sup>-/-</sup>) mice display reduced neointimal formation after arterial injury.<sup>24,30</sup>

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Recently, Bush et al<sup>31</sup> reported that arterial hypertrophy induced by Ang II–induced hypertension was attenuated in CCR2<sup>-/-</sup> mice, suggesting that CCR2 is required for arterial hypertrophy induced in Ang II–induced hypertension. Most previous studies investigating the inflammatory aspects of hypertensive vascular disease, however, focused exclusively on stress- or injury-induced local changes in inflammation-driving factors in arterial wall cells. The functional importance of CCR2 expression on monocytes in hypertension-induced inflammation and vascular remodeling, on the other hand, has received little attention. Therefore, the present study tested the hypothesis that (1) CCR2 expressed in circulating monocytes is enhanced in hypertensive animals and patients via stimulation of Ang II type-1 (AT<sub>1</sub>) receptors; and (2) CCR2 expressed on circulating monocytes has a critical role in hypertension-induced inflammation and vascular remodeling. To dissect the specific role of CCR2 in monocytes, we used bone marrow cells transplantation (BMT) techniques to create mice with a leukocyte-selective CCR2 deficiency (BMT-CCR2<sup>-/-</sup> mice) and demonstrated that Ang II–induced inflammation and vascular remodeling were blunted in BMT-CCR2<sup>-/-</sup> mice as well as in CCR2<sup>-/-</sup> mice.

## Materials and Methods

### Experimental Animals

Male wild-type mice (C57 BL/6J) and those overexpressing human superoxide dismutase (6-TgN(SOD1)3Cje) (SOD-TG) were purchased from Jackson Laboratory (Bar Harbor, Maine). Male AT<sub>1</sub> receptor–deficient mice (AT<sub>1</sub>R-KO) on a C57BL/6J genetic background<sup>32</sup> were supplied from Tanabe Seiyaku Inc., Osaka, Japan. CCR2<sup>-/-</sup> and wild-type (CCR2<sup>+/+</sup>) mice with the same genetic background (C57BL/6J and 129/svjae hybrids) were supplied from Dr Charo.<sup>25</sup> Male CCR2<sup>-/-</sup> and CCR2<sup>+/+</sup> mice were age matched for all experiments. All mice were bred and maintained in the Laboratory of Animal Experiments at Kyushu University. Twenty-week-old male Wistar-Kyoto (WKY) rats and 12-week-old spontaneous hypertensive rats (SHR) were obtained from an established colony at the animal Research Institution of Kyushu University, Faculty of Medical Sciences (Fukuoka, Japan).

### Experimental Protocol

The study protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences. A part of this study was performed at the Kyushu University Station for Collaborative Research and the Morphology Core Unit, Kyushu University Faculty of Medical Sciences.

### Experiment 1

To examine whether Ang II affects CCR2 fluorescence intensity in circulating monocytes *in vivo*, Ang II was infused into several groups of 12-week-old wild-type mice treated with or without AT<sub>1</sub> receptor blocker (ARB), AT<sub>1</sub>R-KO, or SOD-TG mice. The control group received untreated chow and drinking water. The Ang II group received Ang II via an osmotic minipump (Alzet) (1.9 mg/kg per day). The minipump was implanted in the peritoneal cavity under anesthesia with ketamine (80 mg/kg IP) and xylazine (10 mg/kg IP). The Ang II+ARB groups received Ang II by an osmotic minipump and ARB (olmesartan at 0.35 or 3.5 mg/kg per day) in chow. Olmesartan was a gift from Sankyo Pharmaceutical Co (Tokyo, Japan). Treatment with olmesartan was started 3 days before Ang II administration was begun. The AT<sub>1</sub>R-KO+Ang II and SOD-TG+Ang II groups were infused with Ang II by osmotic minipumps.

### Experiment 2

To determine the role of CCR2 in Ang II–induced vascular remodeling, four groups of 12-week-old CCR2<sup>-/-</sup> and CCR2<sup>+/+</sup> mice infused with Ang II or PBS via osmotic minipump were studied.

### Experiment 3

To dissect the specific role of CCR2 in the monocytes, we used the BMT technique to create mice with a leukocyte selective CCR2 deficiency (BMT-CCR2<sup>-/-</sup>). At 8 weeks of age, BMT was performed as described previously.<sup>33</sup> Bone marrow cells were harvested from femurs and tibiae of either test (CCR2<sup>-/-</sup>) or control (CCR2<sup>+/+</sup>) donor mice. The recipient CCR2<sup>+/+</sup> mice received 1×10<sup>7</sup> bone marrow cells (0.3 mL) 4 hours after whole body irradiation with 7 Gy of X-rays (200 KVp, 20 mA, 0.3 mmCu filter) at 1Gy/min. These two groups of mice are referred to as BMT-CCR2<sup>-/-</sup> and BMT-CCR2<sup>+/+</sup>, respectively. Four groups of BMT-CCR2<sup>-/-</sup> and BMT-CCR2<sup>+/+</sup> mice infused with Ang II or PBS were studied. BMT-AT<sub>1</sub>R-KO mice also were created in a same manner. To ensure that the exposure dose was sufficient to ablate the bone marrow, a group of C57BL/6J mice (n=6) was irradiated and injected with bone marrow cells from C57BL/6J mice expressing green fluorescence protein. Flow cytometric analysis of circulating leukocytes from recipient mice at 4 weeks after transplantation revealed that the chimerism was 95±2%.

### Experiment 4

To examine the effect of ARB on monocyte CCR2 fluorescence intensity in other forms of hypertension, we used WKY rats treated with N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME) and SHR treated with or without ARB.

In all experiments, mice were euthanized on day 3, 7, or 28 of treatment for morphometric, immunohistochemical, and biochemical analysis. Venous blood was collected immediately before the mice were euthanized. The aortas and hearts were isolated and either fixed in 10% buffered formalin for histologic analysis or snap-frozen in liquid nitrogen and stored at -80°C for biochemical analysis. Systolic blood pressure was measured by the tail-cuff method before and 3, 7, and 28 days after treatment.

### Histology and Immunohistochemistry

Histopathology and immunohistochemistry were performed as described previously.<sup>24,28</sup> Some sections were subjected to immunostaining using antibodies against mouse macrophages (Mac-3, Serotec Inc), proliferating cell nuclear antigen (PCNA, DAKO), α-smooth muscle cell actin (α-SM actin) (Boehringer Mannheim), MCP-1, and CCR2 (Santa Cruz Biotechnology Inc). The degree of vascular remodeling (the medial thickness and perivascular fibrosis of aorta) and left ventricular hypertrophy [left ventricular to body weight ratios (LV/BW)] on day 28 was measured as described previously.<sup>23</sup>

### TaqMan Real-Time Reverse Transcription–Polymerase Chain Reaction Analysis

TaqMan real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed as previously described.<sup>18</sup> Transcripts from 1 μg total RNA were reverse-transcribed and the resultant cDNA was amplified by TaqMan real-time RT-PCR. The PCR primers for mouse MCP-1, CCR2, and B-type natriuretic peptide (BNP) were sense primer 5'-CCTGGATCGGA-ACCAAATGA-3', antisense primer 5'-CGGGTCAACTTCA-CATTCAAAG-3', and probe oligonucleotides 5'-AACT-GCATCTGCCCTAAGG-TCTTCAGCA-3' for MCP-1, and sense primer 5'-CCTTGGGA-ATGAGTAACTGTGTGAT-3', antisense primer 5'-ATGGA-GAGATACCTTCGGA-CTTCT-3', and probe oligonucleotides 5'-CACTTAGACCAGGCCATGCAGGT-GACA-3' for CCR2, and sense primer 5'-GCCAGTCTCCAG-AGCAATTCA-3', antisense primer 5'-GTGAGGCCTTGG-TCCTTCAA-3', and probe oligonucleotides 5'-TCTCTTATCA-GCTCCAGCAGCTTCTGCA-3' for BNP. GAPDH probe was obtained from Applied Biosystems.

## Flow Cytometry Analysis

Flow cytometry analysis was performed as described previously.<sup>33</sup> To determine CCR2 expression in monocytes, isolated leukocytes were stained using antibodies against phycoerythrin (PE)-conjugated anti-mouse monocyte (CD80) (Becton Dickinson Biosciences), goat anti-mouse CCR2 (Santa Cruz Biotechnology Inc), and FITC-conjugated mouse anti-goat IgG (Santa Cruz Biotechnology Inc.). To determine CCR2 fluorescence intensity in lymphocytes and neutrophils, leukocytes were also stained using antibodies against PE-conjugated anti-mouse CD11b (Mac-1), cy-chrome-conjugated anti-mouse T-cell receptor $\beta$  chain monoclonal antibody (Becton Dickinson Biosciences). In control experiments, FITC-conjugated nonspecific goat IgG was used to measure nonspecific binding. The fluorescent probe, 2',7',-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes Inc), was used to detect intracellular ROS. Isolated leukocytes were stained using DCFH-DA and PE-conjugated anti-mouse CD80 for 30 minutes at 4°C. Stained cells were analyzed by FACSCalibur (Becton Dickinson Biosciences).

## Peripheral Blood Mononuclear Cell Chemotaxis

Mouse mononuclear cells were purified by centrifugation on Lympholyte-M and were washed with RPMI 1640. Cell migration was measured in 96-well chemotaxis chambers (Neuro Probe Inc). MCP-1 in RPMI 1640 (25 ng/mL) was added to the lower and the isolated mononuclear cells ( $2 \times 10^7$ ) in the same medium to the upper wells. After incubation for 90 minutes at 37°C, the membrane was removed, washed on the upper side with PBS. Migrated cells were counted. All assays were performed in triplicate.

## Plasma Measurements

Commercially available ELISA kits (Biosource International) were used to measure mouse MCP-1 according to the manufacturer's instructions.

## Patient Studies

Patients with hypertension who had no other evidence of other cardiovascular disease, infectious disease, inflammatory disorders, connective tissue disease, or prior malignant tumor disease, were enrolled. The patients were divided into three groups: normotensive group (systolic/diastolic blood pressure <140/90 mm Hg), untreated essential hypertensive patients (systolic/diastolic blood pressure  $\geq$ 140/90 mm Hg), and hypertensive patients treated solely with ARB (losartan, candesartan, and valsartan). Hypertensive patients treated with a combination of antihypertensive drugs were not enrolled. Written informed consent was obtained from all patients.

Blood samples were obtained from all patients in the fasting state. White blood cell count, C-reactive protein, and plasma lipid, as well as plasma MCP-1 (human MCP-1 ELISA kit, Biosource International) were measured. The fluorescence intensity of chemokine receptors on leukocytes (the MCP-1 receptor CCR2 on monocytes, the interleukin-8 receptor CXCR2 on neutrophils, and the RANTES receptor CCR1 on T cells, DAKO) also was assessed by flow cytometry.

There were no significant differences in age, body mass index, plasma lipid levels, white blood cell count, or smoking habit among groups (Table 1). Plasma concentrations of MCP-1 and C-reactive protein were lower in the ARB-treated group, although the difference was not statistically significant. As expected, systolic and diastolic blood pressure were significantly higher in untreated hypertensive patients than in normotensive control subjects. Blood pressure values were lower in hypertensive patients treated with ARB than in untreated hypertensive patients, although the difference was not statistically significant. Hypertensive patients treated with ARB had a significantly higher proportion of diabetes mellitus.

## Statistical Analysis

Data are expressed as mean  $\pm$  SE. In animal studies, statistical analysis of differences was compared by ANOVA using Bonferroni's correction for multiple comparisons. In human studies, multiple logistic regression analysis was performed to identify significant

**TABLE 1. Clinical and Biochemical Characteristics of Hypertensive Patients With and Without ARB Treatment and Normotensive Controls**

Variables	Normotensive Controls n=40	Untreated HT n=30	HT Treated With ARB n=30
Age	55 $\pm$ 12	63 $\pm$ 8	59 $\pm$ 11
Male, %	71.7	63.0	63.6
Smoking, %	23.9	40.7	37.3
Body mass index	23 $\pm$ 3	24 $\pm$ 4	23 $\pm$ 3
Hypercholesterolemia, %	34.8	37.0	46.4
Diabetes, %	21.1	29.6	53.7*
Systolic BP, mm Hg	115 $\pm$ 12	151 $\pm$ 10*	143 $\pm$ 17*
Diastolic BP, mm Hg	67 $\pm$ 10	84 $\pm$ 9*	81 $\pm$ 10*
WBC, $\times 10^3/\mu$ L	5.5 $\pm$ 1.6	5.9 $\pm$ 1.6	5.4 $\pm$ 2.0
Monocyte count, $\times 10^3/\mu$ L	314 $\pm$ 116	302 $\pm$ 115	262 $\pm$ 122
Total cholesterol, mg/dL	205 $\pm$ 43	203 $\pm$ 36	216 $\pm$ 37
Triglycerides, mg/dL	120 $\pm$ 57	119 $\pm$ 50	142 $\pm$ 53
LDL-C, mg/dL	112 $\pm$ 33	108 $\pm$ 34	114 $\pm$ 21
C-reactive protein, mg/dL	0.13 $\pm$ 0.4	0.17 $\pm$ 0.3	0.10 $\pm$ 0.3
MCP-1, pg/mL	11 $\pm$ 6	13 $\pm$ 10	9 $\pm$ 6
CCR2 in monocytes, MFI	115 $\pm$ 32	168 $\pm$ 37*	111 $\pm$ 24†
CXCR2 in neutrophils, MFI	85 $\pm$ 35	77 $\pm$ 25	81 $\pm$ 16
CCR1 on T cells, MFI	10 $\pm$ 3	11 $\pm$ 3	10 $\pm$ 3

Values are mean  $\pm$  SEM. \* $P$ <0.01 vs normotensive controls, † $P$ <0.01 vs untreated hypertensive patients (HT).

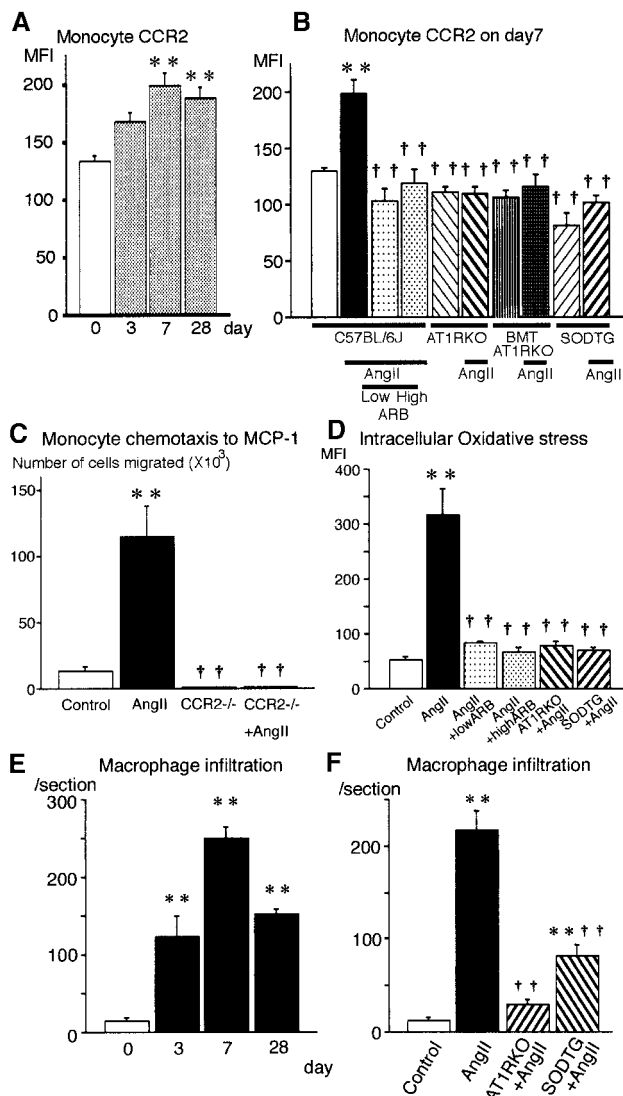
independent predictors of the presence of hypertension. Odds ratio and 95% confidence intervals were calculated. A level of  $P$ <0.05 was considered statistically significant.

## Results

### Ang II–Induced Upregulation of CCR2 in Circulating Monocytes in Wild-Type Mice

Flow-cytometry analysis indicated that monocyte CCR2 intensity peaked on day 7 and the higher level was sustained until day 28 of Ang II infusion (Figure 1A). The Ang II–induced increase in CCR2 intensity on day 7 was prevented by treatment with low and high doses of ARB. The Ang II–induced increase in CCR2 intensity was blunted in AT<sub>1</sub>R-KO, SOD-TG, and BMT-AT<sub>1</sub>R-KO mice (Figure 1B). No CCR2 antigen was detected on lymphocytes or neutrophils in the presence or absence of Ang II infusion. To determine the functional role of the CCR2 upregulation in monocytes, chemotaxis assay was performed in peripheral blood mononuclear cells. No chemotactic response was detected in monocytes from control untreated mice. In contrast, MCP-1–mediated chemotaxis was increased in monocytes from mice infused Ang II for 7 days, which was blunted in monocytes from CCR2<sup>-/-</sup> mice (Figure 1C). These chemotaxis data are in agreement with previous studies by other investigators<sup>34,35</sup> that the measured increase in CCR2 intensity on monocytes resulted in increased monocyte chemotaxis in response to MCP-1.

To study the effects of Ang II on intracellular ROS, intracellular ROS were measured in monocytes by



**Figure 1.** Ang II-induced CCR2 expression on circulating monocytes and macrophage infiltration into the aorta in wild-type mice. **A**, Time course of mean fluorescence intensity (MFI) of CCR2 on the surface of the circulating monocytes;  $n=6$  to  $8$ . **B**, Effects of ARB, AT<sub>1</sub> deficiency, leukocyte-selective AT<sub>1</sub>R deficiency, and SOD overexpression on Ang II-induced enhancement of CCR2 expression on day 7;  $n=7$  each. **C**, Effects of increased CCR2 expression on peripheral mononuclear cells chemotaxis in response to MCP-1 (25 ng/mL) in wild-type and CCR2<sup>-/-</sup> mice infused with or without Ang II for 7 days. **D**, Effects of ARB, AT<sub>1</sub> deficiency, and SOD overexpression on intracellular production of ROS in monocytes;  $n=6$  each. **E**, Time course of macrophage infiltration after Ang II infusion (number of Mac-3-positive macrophages per section);  $n=6$  each. **F**, Effect of ARB, AT<sub>1</sub> deficiency, and SOD overexpression on Ang II-induced macrophage infiltration into the aorta. Number of Mac-3-positive macrophages per section was determined on day 7;  $n=6$  each. \* $P<0.05$ , \*\* $P<0.01$  vs control group; † $P<0.05$ , †† $P<0.01$  vs Ang II group.

DCFH-DA fluorescence intensity (Figure 1D). Levels of ROS were undetectable in monocytes from untreated control mice, but were significantly increased in monocytes from mice infused with Ang II for 7 days. Treatment with the low and high doses of ARB prevented the increase in intracellular

ROS. The Ang II-induced increase in ROS was blunted in AT<sub>1</sub>R-KO and SOD-TG mice.

The time course of macrophages infiltration into the aorta was similar to that of monocyte CCR2 expression after Ang II infusion (Figures 1E and 1F). There was reduced Ang II-induced monocyte infiltration into the aorta in mice treated with low and high doses of ARB, AT<sub>1</sub>R-KO, and SOD-TG mice.

Compared with the control group, Ang II infusion induced a rise in systolic blood pressure (online Table 1, available in the online data supplement at <http://circres.ahajournals.org>). Similar changes in blood pressure were observed in Ang II+Low ARB and SOD-TG+Ang II groups. No Ang II-induced increase in blood pressure was observed in the Ang II+High ARB and AT<sub>1</sub>R-KO+Ang II groups (online Table 2). Baseline blood pressure was lower in AT<sub>1</sub>R-KO mice, although the difference was not statistically significant.

### Ang II-Induced Upregulation of MCP-1 and CCR2 in the Aorta in Wild-Type Mice

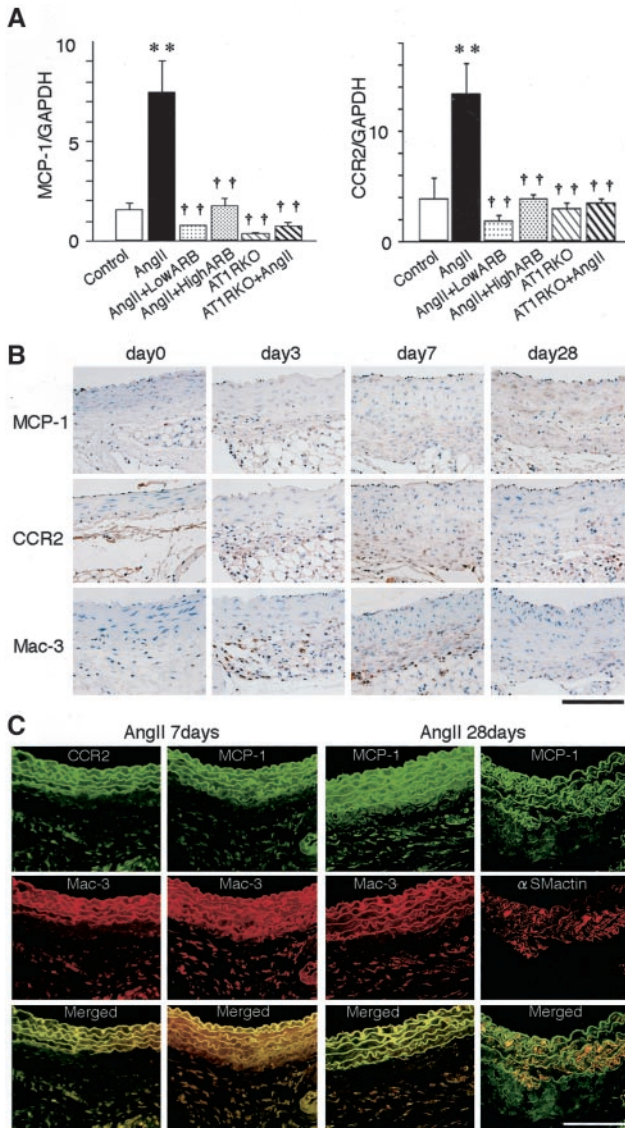
As previously reported,<sup>12,31</sup> Ang II infusion to wild-type mice for 7 days increased mRNA levels of MCP-1 and CCR2 (Figure 2A). Treatment with low and high doses of ARB prevented the increased gene expression. The Ang II-induced increase in gene expression was blunted in AT<sub>1</sub>R-KO and SOD-TG mice. Immunohistochemical staining indicated that Ang II infusion increased MCP-1 in lesional macrophages on days 3 and 7, and the increase was sustained until day 28 (Figures 2B and 2C). Increased MCP-1 staining was noted in smooth muscle cells of the media on day 28. Increased CCR2 staining was observed in infiltrating macrophages on days 3, 7, and 28. Treatment with low and high doses of ARB prevented the Ang II-induced increase in immunostaining for MCP-1 and CCR2 (data not shown).

### Inhibition of Ang II-Induced Vascular Remodeling in CCR2<sup>-/-</sup> Mice

As previously reported,<sup>31</sup> Ang II infusion to CCR2<sup>+/+</sup> mice for 7 days induced infiltration of Mac-3-positive macrophages into the aortic wall, mainly into the adventitia (Figure 3A). PCNA-positive proliferating cells appeared in cells in the endothelial layer, media, and adventitia. There were also  $\alpha$ -SM actin-positive cells (myofibroblast) in the adventitia of Ang II-infused wild-type mice (data not shown). On day 28, aortic remodeling (medial wall thickening and perivascular fibrosis) developed in CCR2<sup>+/+</sup> mice. In contrast, aortic inflammatory-proliferative changes in the early stage and vascular remodeling in the late stage were markedly attenuated in CCR2<sup>-/-</sup> mice (Figure 3B).

There were no significant differences in plasma MCP-1 levels between untreated CCR2<sup>+/+</sup> and CCR2<sup>-/-</sup> mice on day 28 (online Table 2). In contrast, the plasma MCP-1 level dramatically increased in CCR2<sup>-/-</sup> mice infused with Ang II, compared with that in CCR2<sup>+/+</sup> mice infused with Ang II.

There were no significant differences in Ang II-induced changes in systolic blood pressure or left ventricular hypertrophy (online Table 2).



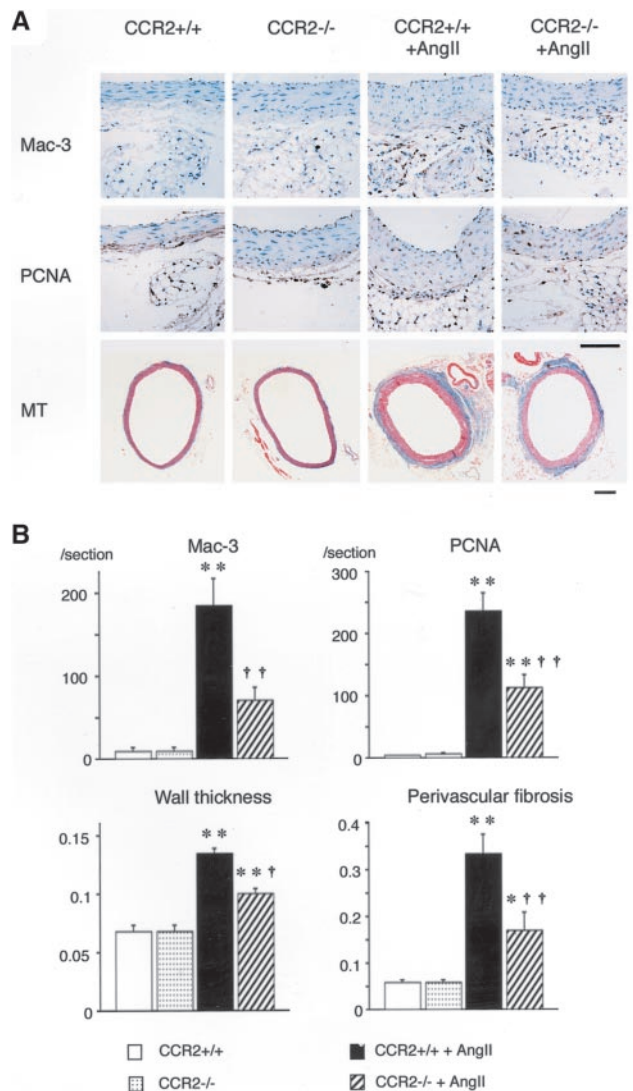
**Figure 2.** Ang II-induced expression of MCP-1 and CCR2 in the aorta in wild-type mice. A, MCP-1 and CCR2 gene expression by real-time RT-PCR in the aorta. Data are expressed as the ratio of MCP-1 and CCR2 mRNA to GAPDH mRNA. \* $P < 0.05$ , \*\* $P < 0.01$  vs control group; † $P < 0.05$ , †† $P < 0.01$  vs Ang II group;  $n = 6$  each. B, Aortic cross sections were stained immunohistochemically for MCP-1, CCR2, and macrophage (Mac-3) on days 0, 3, 7, and 28 ( $n = 6$  each) (bar = 100  $\mu\text{m}$ ). C, Immunofluorescence double staining of aorta using anti-mouse CCR2, MCP-1, Mac-3, or  $\alpha$ -SM actin antibody. CCR2 and MCP-1 immunofluorescence (labeled green with FITC), Mac-3 and  $\alpha$ -SM actin immunofluorescence (labeled red with rhodamine), and their merged images (yellow) are presented. Background nonspecific fluorescence is observed in elastic layers of aortas (bar = 100  $\mu\text{m}$ ).

**Blunted Ang II-Induced Vascular Remodeling in BMT-CCR2<sup>-/-</sup> Mice**

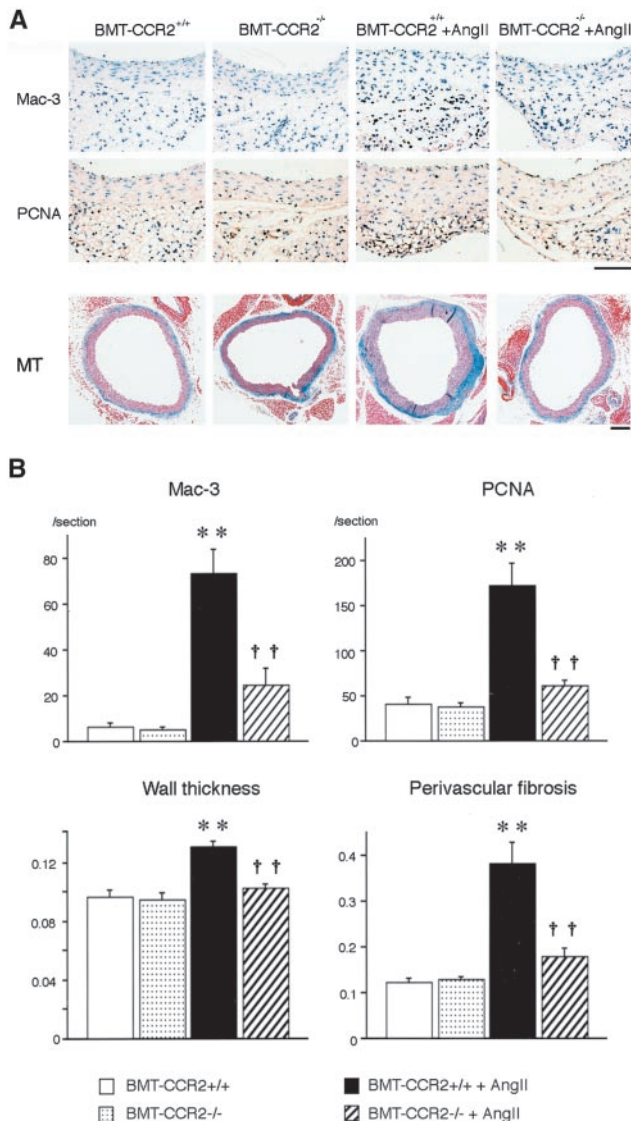
There were no differences in the degree of aortic wall thickening or perivascular fibrosis between untreated BMT-CCR2<sup>+/+</sup> and BMT-CCR2<sup>-/-</sup> mice. BMT-CCR2<sup>+/+</sup> mice developed significant vascular remodeling and left ventricular hypertrophy in response to Ang II infusion to an extent similar to that in nonirradiated CCR2<sup>+/+</sup> mice. In contrast, Ang II-induced aortic wall thickening and perivascular fibro-

sis were blunted in BMT-CCR2<sup>-/-</sup> mice (Figures 4A and 4B). There were no significant differences in Ang II-induced hypertension, left ventricular hypertrophy, or the increase in BNP mRNA levels between BMT-CCR2<sup>+/+</sup> and BMT-CCR2<sup>-/-</sup> mice (online Table 3).

As in nonirradiated CCR2<sup>-/-</sup> mice, there was a significant increase in plasma MCP-1 levels only in BMT-CCR2<sup>-/-</sup> mice infused with Ang II (online Table 3). Ang II-induced increases in MCP-1 mRNA and immunoreactive MCP-1 levels in the aorta were also similar between BMT-CCR2<sup>+/+</sup> and BMT-CCR2<sup>-/-</sup> mice (Figure 5). Aortic CCR2 gene expression was blunted in BMT-CCR2<sup>-/-</sup> mice infused with and



**Figure 3.** Inhibition of Ang II-induced inflammation and vascular remodeling in CCR2<sup>-/-</sup> mice. A, Aortic cross sections were immunohistochemically stained for macrophages (Mac-3) or a marker of proliferation (PCNA) on day 7. Aortic sections stained with Masson's trichrome on day 28 are also shown (bar = 100  $\mu\text{m}$ ). B, Number of macrophages infiltrated into the aorta and appearance of proliferating cells in the CCR2<sup>+/+</sup>, CCR2<sup>-/-</sup>, CCR2<sup>+/+</sup> + Ang II, and CCR2<sup>-/-</sup> + Ang II mice on day 7 of Ang II infusion are shown (positive cell counts per section). Wall thickness (wall-to-lumen ratio) and perivascular fibrosis of aorta on day 28 are shown. \* $P < 0.05$ , \*\* $P < 0.01$  vs CCR2<sup>+/+</sup> group; † $P < 0.05$ , †† $P < 0.01$  vs CCR2<sup>+/+</sup> + Ang II group ( $n = 6$  each).



**Figure 4.** Blunted Ang II-induced inflammation and vascular remodeling in BMT-CCR2<sup>-/-</sup> mice. A, Aortic cross sections were immunohistochemically stained for macrophages (Mac-3) or a marker of proliferation (PCNA) on day 28. Aortic sections stained with Masson's trichrome on day 28 are also presented (bar=100 μm). B, Number of macrophages infiltrated into the aorta and appearance of proliferating cells in the BMT-CCR2<sup>+/+</sup>, BMT-CCR2<sup>-/-</sup>, BMT-CCR2<sup>+/+</sup>+Ang II, and BMT-CCR2<sup>-/-</sup>+Ang II mice of day 28 of Ang II infusion are shown (positive cell counts per section). Wall thickness (wall-to-lumen ratio) and perivascular fibrosis of the aorta on day 28 are also shown. \**P*<0.05, \*\**P*<0.01 vs BMT-CCR2<sup>+/+</sup> group; †*P*<0.05, ††*P*<0.01 vs BMT-CCR2<sup>+/+</sup>+Ang II group (n=6 each).

without Ang II, suggesting that lesional macrophages might be the major source of CCR2 gene expression in the aortic tissue.

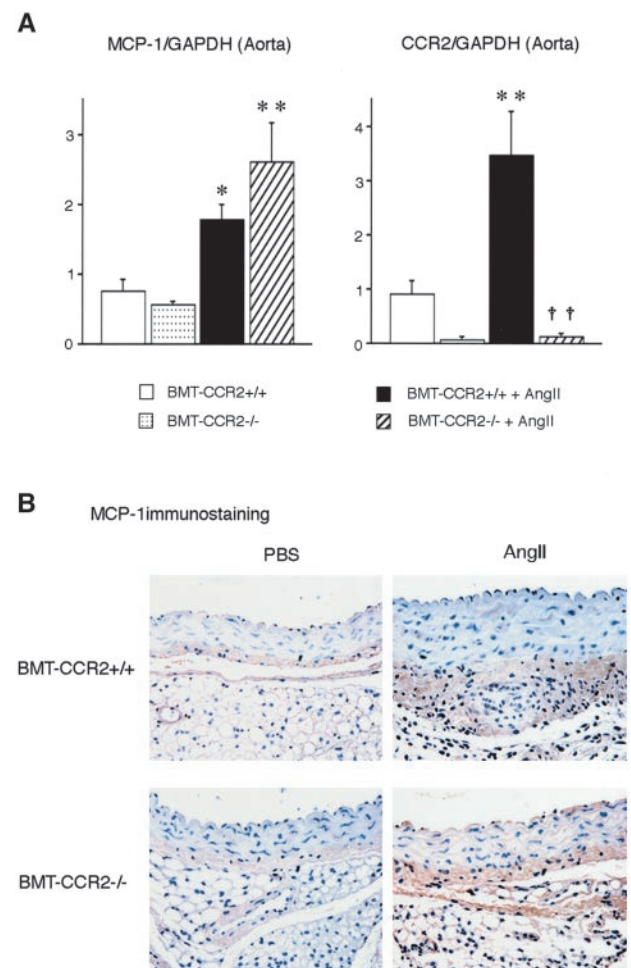
### Effects of ARB on CCR2 Expression in Circulating Monocytes in Hypertensive Patients

A pilot clinical study was performed to determine if monocyte CCR2 is enhanced in patients with essential hypertension via AT<sub>1</sub> receptor stimulation. Multivariate analysis revealed that the increased CCR2 intensity on monocytes

correlated independently with the presence of hypertension (odds ratio=33.6, 95% confidence interval=4.22~267.5; *P*<0.01; Table 2). The monocyte CCR2 intensity in hypertensive patients treated with ARB was similar to that observed in normotensive subjects (Table 1). There were no significant differences in monocyte CCR2 levels in patients treated with different ARB (data not shown). In contrast, there were no significant differences in CXCR2 in neutrophils, or CCR1 in T cells among the three groups (Table 1). There was no significant correlation between the CCR2 intensity on monocytes and the degree of systolic or diastolic hypertension (data not shown).

### CCR2 Upregulation in Peripheral Circulating Monocytes of Hypertensive Rats

Monocyte CCR2 intensity was higher in WKY rats made hypertensive with administration of the nitric oxide synthesis inhibitor (L-NAME) for 1 week<sup>23</sup> compared with control



**Figure 5.** MCP-1 and CCR2 expression of the aorta in BMT mice. A, MCP-1 and CCR2 gene expression by real-time RT-PCR in the aorta on day 28. Data are expressed as the ratio of MCP-1 and CCR2 mRNA to GAPDH mRNA. \**P*<0.05, \*\**P*<0.01 vs BMT-CCR2<sup>+/+</sup> group; †*P*<0.05, ††*P*<0.01 vs BMT-CCR2<sup>+/+</sup>+Ang II group (n=6 each). B, Aortic cross sections were stained immunohistochemically for MCP-1 on day 28 (bar=100 μm).

**TABLE 2. Identification of Independent Predictors of the Presence of Hypertension by Multivariate Analysis**

Variables	Odds Ratio	95% CI	P
Nonpredictor			
Sex, male	6.02	0.77–47.4	0.40
Age, >70 years	2.24	0.26–19.5	0.47
Smoking	3.49	0.41–29.5	0.25
Alcohol	0.80	0.11–5.82	0.83
Body mass index, >25	6.46	0.55–75.2	0.14
Hypercholesterolemia			
TC, >220 mg/dL	0.28	0.03–2.63	0.26
HDL-C, <40 mg/dL	0.37	0.44–3.51	0.39
LDL-C, >130 mg/dL	0.88	0.07–11.3	0.92
Diabetes	1.78	0.18–17.9	0.63
MCP-1, <8 ng/mL	0.54	0.10–3.04	0.49
CXCR2/neutrophils, MFI	0.98	0.94–1.01	0.15
CCR2/T cells, MFI	0.99	0.74–1.32	0.95
Predictor			
CCR2/monocytes, >130, MFI	33.6	4.22267.5	<0.01

untreated rats. The increase in monocyte CCR2 intensity was prevented by treatment with low and high doses of ARB. Monocyte CCR2 intensity was also enhanced in 12-week old SHR, which was reduced by treatment with low and high doses of ARB (online Table 4).

### Discussion

Hypertension-induced mechanical strains on monocytes and endothelial cells activate monocyte function resulting in hyperresponsiveness to inflammatory stimuli and secretion of cytokines and growth factors.<sup>36–38</sup> The novel findings of the present study are that (1) CCR2 expression and function (chemotaxis to MCP-1) are enhanced in circulating monocytes in hypertensive animals through an AT<sub>1</sub> receptor-mediated mechanism, and (2) monocyte CCR2 is critical for monocyte-mediated inflammation and remodeling in Ang II-induced hypertension in mice. Although the precise molecular mechanism underlying AT<sub>1</sub> receptor-mediated overexpression of CCR2 in the monocytes is not known, the present data using the SOD-TG mice suggest the possible involvement of ROS. The involvement of ROS is supported by a recent report by Wang et al<sup>35</sup> who demonstrated that treatment of isolated monocytes with antioxidants reduced homocysteine-induced increases in CCR2.

The pilot clinical study indicating that (1) increased CCR2 on monocytes is an important predictor of the presence of hypertension, and (2) hypertensive patients treated with ARB have reduced levels of monocyte CCR2 suggests potential clinical implications of the present observation in animals. Our present data of (1) identical effects of treatment with low and high doses of ARB on monocyte CCR2 expression in mice and rats, and (2) no significant correlation between blood pressure values and the monocyte CCR2 intensity in patients support the hypothesis that ARB-induced diminished CCR2 expression is not explained solely by the blood pressure-lowering effects of ARB. Therefore, a direct action

of Ang II mediated via AT<sub>1</sub> receptors beyond blood pressure lowering might be involved in the pathobiology of enhanced monocyte CCR2 expression and function under hypertensive conditions in vivo.

To dissect the relative pathobiological role of monocytes versus nonmonocyte cells in the arterial wall, we used the BMT technique. The most important finding of this study was blunted Ang II-induced aortic inflammation and remodeling in BMT-CCR2<sup>-/-</sup> mice as well as in CCR2<sup>-/-</sup> mice. In contrast, cardiac hypertrophy developed in both CCR2<sup>-/-</sup> and BMT-CCR2<sup>-/-</sup> mice. The present study therefore provides direct evidence that increased CCR2 expression on monocytes is critical for the development of vascular inflammation and remodeling, but not for left ventricular hypertrophy, in Ang II-induced hypertension. It is plausible therefore that suppression of Ang II-induced vascular remodeling by leukocyte-selective CCR2 deficiency may be the result of reduced recruitment and activation of monocytes. Activated leukocytes might produce growth-promoting signals, which in turn lead to vascular remodeling in a CCR2-deficiency manner. In contrast, Roque et al<sup>30</sup> suggested that upregulation of MCP-1 is responsible for attenuating neointimal smooth muscle growth after arterial injury in CCR2<sup>-/-</sup> mice. Although there was no detectable difference in Ang II-induced local MCP-1 expression between BMT-CCR2<sup>+/+</sup> and -CCR2<sup>-/-</sup> mice, it is possible that some of inhibitory effects seen in BMT-CCR2<sup>-/-</sup> mice might be related to direct inhibitory effects of MCP-1. The blunted Ang II-induced inflammation and vascular remodeling in BMT-CCR2<sup>-/-</sup> mice cannot be explained by nonspecific action of irradiation, because (1) Ang II-induced structural changes in the aorta and heart were similar between CCR2<sup>+/+</sup> and BMT-CCR2<sup>+/+</sup> mice and (2) Ang II-induced increases in gene and protein expression of MCP-1 were noted in CCR2<sup>-/-</sup> and BMT-CCR2<sup>-/-</sup> mice. In conclusion, the present study provides solid evidence that CCR2 expressed on monocytes has a critical role in vascular inflammation and remodeling in Ang II-induced hypertension. This finding might also apply to the pathology of vascular remodeling due to other types of hypertension, because enhanced CCR2 expression and its inhibition by ARB treatment was demonstrated in other types of hypertensive animals and in hypertensive patients. The present data suggest that CCR2-mediated monocyte inflammation is a reasonable target of therapy for treatment of vascular remodeling. Also, blockade of Ang II signals by ARB might act as antiinflammatory therapy beyond blood pressure lowering.

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## Critical Role of Monocyte Chemoattractant Protein-1 Receptor CCR2 on Monocytes in Hypertension-Induced Vascular Inflammation and Remodeling

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## Online Materials

**Online Table 1.** Systolic blood pressure in Experiment 1

Groups	Systolic blood pressure (mmHg)		
	Day 0	Day 7	Day 28
Control	130±8	134±5	136±7
AngII	133±6	182±10**	187±11**
AngII +Low ARB	130±6	183±9**	NE
AngII +High ARB	129±5	142±6††	NE
AT1R-KO	100±9	103±7††	104±7††
AT1R-KO +AngII	104±7	110±5††	106±9††
SOD-TG	131±4	130±6	134±7
SODTG +AngII	129±3	172±5**	178±8**

Values are mean ± SE. \*P <0.05, \*\*P<0.01 versus corresponding controls. †P <0.05, ††P <0.01 versus AngII infused group. n=6 each. NE= not examined.

**Online Table 2.** Systolic blood pressure, plasma MCP-1, left ventricular to body weight ratio in Experiment 2.

Groups	Systolic blood pressure (mmHg)			MCP-1 (pg/ml)	LV/BW (mg/g)
	Day 0	Day 7	Day 28	Day28	Day28
CCR2 <sup>+/+</sup>	134±7	136±6	132±8	42±6	4.5±0.1
CCR2 <sup>-/-</sup>	131±8	128±6	137±6	47±9	4.5±0.1
CCR2 <sup>+/+</sup> +AngII	135±8	183±5**	220±11**	40±2	5.0±0.1**
CCR2 <sup>-/-</sup> +AngII	130±8	188±10**	213±7**	118±30**	5.1±0.2**

Values are mean ± SE. \*P < 0.05, \*\*P < 0.01 versus corresponding controls. †P < 0.05, ††P < 0.01 versus AngII infused group. n=6 each.

**Online Table 3.** Systolic blood pressure, plasma MCP-1, left ventricular to body weight ratio, and BNP in Experiment 3.

Groups	Systolic blood pressure (mmHg)			MCP-1 (pg/ml)	LV/BW (mg/g)	BNP mRNA (BNP/GAPDH)
	Day 0	Day 7	Day 28	Day28	Day28	Day28
BMT-CCR2 <sup>+/+</sup>	123±4	124±3	128±4	36±6	4.1±0.1	1.7±0.1
BMT-CCR2 <sup>-/-</sup>	121±4	129±4	130±5	48±5	4.0±0.1	1.8±0.1
BMT-CCR2 <sup>+/+</sup> +AngII	122±3	191±8**	194±7**	39±2	5.8±0.4**	3.6±0.7*
BMT-CCR2 <sup>-/-</sup> +Ang	125±2	189±5**	196±5**	97±12**	5.8±0.3**	3.4±0.3*

Values are mean ± SE. \*P < 0.05, \*\*P < 0.01 versus corresponding controls. †P < 0.05, ††P < 0.01 versus AngII infused group. n=6 each.

**Online Table 4.** Systolic blood pressure, CCR2/Monocyte (MFI) in Experiment 4.

Groups	Systolic blood pressure (mmHg)	CCR2/Monocyte (MFI)
WKY	130±7	104±5
L-NAME	173±10	151±7
L-NAME +low ARB	169±6	80±8
L-NAME +high ARB	139±6	77±5
SHR	178±11	145±9
SHR +low ARB	169±8	90±8
SHR +high ARB	135±10	93±9

Values are mean ± SE. \*P < 0.05, \*\*P < 0.01 versus corresponding controls. †P < 0.05, ††P < 0.01 versus AngII infused group. n=6 each.